Avian Diseases

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THE USE OF THE SERUM SPOT PLATE TEST FOR AVIAN PPLO

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OF THE recently developed tests for the demonstration of antibody to pleuropneumonia-like organsims (PPLO) [1,3,4,5,6], we have selected the serum plate agglutination test because it is reasonably simple, rapid, sensitive and specific. During the past 3 years we have tested approximately 30,000 serum samples by this method. This report describes the methods and materials employed and summarizes some of our observations and experiences in using the serum plate test.

MATERIALS

Serum Specimens: Blood samples were obtained by cardiac puncture and transfer of 2 to 3 ml of blood to 12 x 75 mm tubes held upright in wire racks. After clotting, the blood samples were separated from the sides of the tube with wooden applicators and then held at 25 to 27 C long enough to obtain a minimum of 0.5 ml of serum. Serum samples were then held at 4 C until tested.

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Serum samples that have been held for more than 3 to 4 days at 4 C or that have been frozen and thawed may contain precipitates (presumably lipids) that markedly interfere with the reading of the serum plate tests. Such samples are centrifuged lightly, and the clear serum withdrawn for testing. Contaminated or gelled samples are obviously unfit for testing.

Heat inactivation of a positive PPLO serum at 56 C for 30 minutes does not destroy its ability to agglutinate the diagnostic antigen under the conditions of our testing procedures. It does tend to reduce the size of the agglutinated aggregates and may delay the time before agglutination, but the quantitative endpoint of a positive serum remains constant with or without inactivation. Likewise, freezing and thawing of serum samples does not alter the agglutination reaction, but checks for the presence of serum precipitates must be made.

Antigen: Multiple, large volume culture flasks, containing identical components of media, serum, PPLO inoculum, etc., have often given an end product that varied greatly in the apparent number of organisms present from one flask to the next. Furthermore a standard nephelometric reading on a given series of broth cultures does not necessarily mean uniform agglutinating capacity. Therefore, in an effort to overcome such variations, we have reported to serological standardization of the final antigen. This approach has given us greater uniformity of antigen reaction and insures a common agglutination capacity from one antigen lot to another.

The serum plate diagnostic antigen is prepared from broth cultures of PPLO isolated from field cases of chronic respiratory disease (CRD) of chickens or infectious sinusitis of turkeys. This antigen is a stained culture concentrate that is standardized serologically against a known positive serum pool in the following manner:

Serial two-fold dilutions of the positive serum pool are made in normal horse serum. The dilution of antigen concentrate that gives an unequivocal agglutination reaction in the 1:8 dilution of the positive serum within 5 minutes is

regarded as the working dilution of antigen. Batches of antigen so standardized, have maintained their agglutinating titer for as long as 25 months.

Performance of the tests: Primary emphasis should be placed on clean glassware and adequate rinsing after washing. Some detergents tend to leave a film on test tubes and syringes that can only be removed by a minimum of three rinsings in clear water. Such a film can even prevent proper clotting of blood samples. The test plate should be washed with pumice soap to remove all wastes, carefully rinsed with hot, then cold water and dried with an absorbent towel.

The routine serum plate test is performed at room temperature using a glass plate measured off in one-inch squares. Clear serums are transferred by medicine dropper, delivering 0.03 to 0.05 ml per drop, to the center of the squares. The dropper used for the test serums is rinsed twice in physiological saline between samples and drain-dried on cotton-gauze sponges. A similar quantity of PPLO antigen is added to each serum sample and mixed with a wooden applicator. For qualitative testing of a large number of samples at one time, two drops of serum to one drop of antigen may be used. In this manner, as many as 45 sera can be tested at one time without drying of the serum-antigen mixture.

After the serum and antigen are mixed, the test plate is gently tilted and rotated while being inspected with the aid of a gooseneck lamp or similar source of light. One helpful practice is to scan the entire plate over the light during the first 15 seconds of rotation to detect any samples that appear to have aggregates already forming. This is a final check for serum precipitates, dust or other foreign material which, if necessary, can be removed by centrifugation of the serum before retesting. Reactions are judged by comparison with known positive and negative reference serums. Strong positive reactions appear within 2 minutes and are indicated by distinct clumping of the antigen and clearing of the serum-antigen mixture. Weak reactions are slower and may take the full 5 minutes reading time. In such specimens, the antigen aggregates appear considerably smaller. The interpreta-

tion of weak reactions requires constant checking against known control serums. When known positive serums are diluted out to an endpoint, the interval before agglutination becomes apparent, and the relative size of the aggregates at the higher dilutions are comparable to those observed in undiluted weakly positive serums. Therefore, two-fold serial dilutions of a given, strongly positive serum, when viewed over the light, will provide a basis for distinguishing weakly positive reactions.

A semi-quantitative test may be performed by the use of 0.2 ml pipettes. Clear serum in amounts of 0.04, 0.02, 0.01 and 0.005 ml are transferred to four separate squares, and 0.04 ml of antigen are added and mixed. This gives dilutions designated as "undiluted, 1:2, 1:4 and 1:8." The reactions are read at the 5 minute interval. If positive reactions are obtained at the 1:8 dilution, a four-fold dilution of the original serum is made and retested as above, giving dilutions designated as "1:4 through 1:32." Because of the small volumes of fluid involved, it is not advisable to set up more than 5 serums at one time.

RESULTS

Field studies of experimental flocks: In 1956, attempts were made to obtain PPLO free chicks by a breeder-antibiotic treatment program coupled with serologic followup of the progeny. A flock of Single Comb White Leghorn breeders, 50 percent of which were positive to the PPLO serum plate test, was selected for this experiment. Twenty-two hundred 38-week-old breeders were injected intramuscularly with a combined penicillin-streptomycin mixture at concentrations of 250,000 units of penicillin and 250 mg streptomycin per bird. Hatching eggs were collected in the interval between 9 and 16 days thereafter, dipped in 300 parts per million of Roccal solution and placed in a separate incubator. The hatched chicks were housed in new quarters which consisted of three pens with capacities of 750 chicks per pen. Vaccinations were done at 3 days and 3 weeks of age with combination Newcastle disease-infectious bronchitis (ND-IB) dust vaccine and at

12 weeks with fowl pox vaccine. This hatch was discarded for further study because 20 percent of the birds gave positive reactions to the serum plate test for PPLO at 12 weeks of age.

The same breeders were injected a second time with streptomycin only (250 mg/hen and 300 mg/rooster), and eggs were collected as in the previous trial. A total of 4257 chicks were segregated in nine buildings with capacities of 300 to 900 chicks each. These shed-roof type buildings had 20 by 20 foot wooden pen divisions, concrete floors, and were equipped with gas brooders. Strict sanitary precautions were observed by all personnel in order to maintain each building as a separate unit. The second progeny group was vaccinated in the same manner as the first hatch and, in addition, received laryngotracheitis (LN) vaccine at 17 weeks of age.

Checks for PPLO agglutinins in the entire flock were conducted at various intervals as shown in table 1. After each bleeding, positive or questionable birds were removed from the flock. The only respiratory symptoms observed throughout this study were the usual "takes" following ND-IB vaccination and slight to moderate coughing in some buildings after LN vaccination. The LN vaccination stress may have been severe enough to facilitate contact spread of latent PPLO, since 17 percent of the birds were found to be PPLO positive on subsequent testing. However, in spite of this apparent spread of infection, one pen remained negative throughout the entire testing program.

Previous experiments, utilizing the experimental antigen to assay for PPLO agglutinins in growing birds, had shown a

TABLE I

PPLO test results on the second progeny group

Age of birds (in weeks)	% of flock positive (PPLO-SP Test)	Range of % Positive among 9 houses
8-9	1.5	0-2.6
11-12	0.75	0-3.2
16-17	0.62	0-1.0
27-28	17.0	3-27.8

wide range in the intensity of reactions on the serum plate test. The significance of weak agglutination reactions encountered had been in doubt, and further studies were considered necessary. Therefore, at the 8 and 12 week bleedings, 64 birds, whose sera had given weakly positive or questionable reactions on the serum plate test, were removed from this flock to clean colony houses at a different location for more detailed study.

It was decided to utilize the semi-quantitative PPLO serum plate test for the study of (1) the maintenance of antibody levels in PPLO positive birds, (2) the appearance of agglutinins in non-reactors held in contact with positive birds, and (3) the relationship of the serologic status of dams to that of their progeny.

All birds were retested periodically and at 5 months of age they were grouped according to their reactions to the PPLO serum plate test. One group located in colony house #11 contained 30 pullets and 3 cockerels. The undiluted serums of these birds, or dilutions no greater than 1:4, gave positive reactions on the serum plate test. The second group, in colony house #12, contained 27 pullets and 4 cockerels. Eight of these pullets had titers of 1:8 or more, and the remaining 19 pullets and 4 cockerels had reactions that were negative or questionable.

After being placed in their respective colony houses, the birds were bled three times at approximately 2 month intervals. During the first 3 days following the first and second bleedings the birds were trap-nested to collect hatching eggs. The chicks were "pedigree" hatched and then bled either at 3 or 6 days of age for serologic tests. For comparative purposes, Newcastle disease-hemagglutination inhibition as well as PPLO serum plate tests were done. The serologic summary of the findings on the 3 sets of serum specimens from the dams of colony house #11 is presented in figure 1. This house contained birds that were all PPLO positive at the start. Over the 6 month test period, some birds, even of those that originally had titers as high as 1:4, eventually became negative. Analysis of the titers for individual birds in this

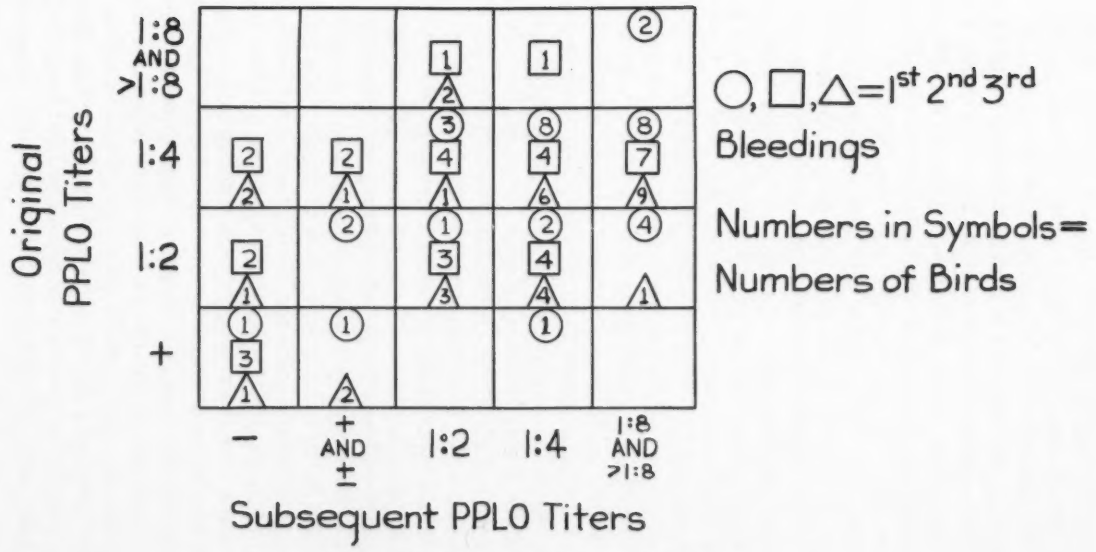

Fig. 1. PPLO titers of house #11 dams.

house, as observed in 132 tests, showed only four instances in which a single test result was at variance with the serologic trend, characteristic of the individual bird.

Figure 2 presents the serologic summary of the findings on the three sets of serum specimens from the dams of colony house #12. This house contained, at the start, the strongly positive and the negative or questionable reactors. It is interesting to note that the stronger positives tended to maintain their status over the 6 month test period. A second point, not shown by the figure, is the fact that two of the four negative cockerels in this group became strong positives. It seems likely that contact transmission occurred under these conditions in colony house #12 since 48 percent of the negative and questionable reactors became positive by the end of the experiment. Again, of 124 tests on these birds during the 6 month period, only one test was at variance with the serologic trend characteristic of the individual bird.

In figure 3, data are presented on the passive transfer of PPLO antibody from dam to chick. The first observation of importance in evaluating the validity of the serum plate test on chick serum is that all chicks from negative dams were also negative. Only three chicks had titers higher than those of their dams. The principal point of interest in these titers is that the level of antibody in the chick is generally lower than that of its dam. In summary, approximately 73 percent of the serum samples from chicks whose dams had titers of 1:4 or less were PPLO negative, whereas roughly 55 percent of those from dams whose titers were 1:8 or higher were positive. For comparative purposes the Newcastle disease hemagglutination-inhibition (H-I) titers on the serum samples from all of the dams and their progeny are summarized in figure 4. Here it may be seen that, as in the case of the PPLO reactors, all of the chicks from H-I negative dams were H-I negative. While 73 percent of the chicks from dams with H-I titers of 1:8 or 1:16 were negative, 92 percent of the chicks from dams with H-I titers of 1:32 or more were positive. The Newcastle disease H-I test is considerably more sensitive than the PPLO serum plate test. Nevertheless, the comparison suggests that

Fig. 2. PPLO titers of house #12 dams.

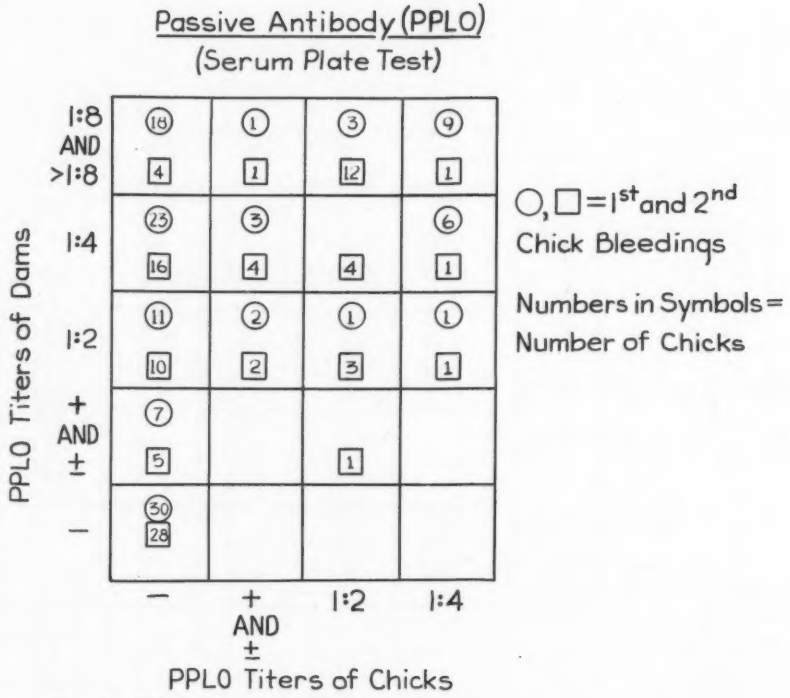

Fig. 3. PPLO titers of chicks and dams.

low levels of PPLO or Newcastle antibodies in the dam are associated with minimal transfer of passive antibody and hence a high percentage of the progeny from such layers will appear as serologic negatives. Further, and more important, weak positives in chicks may be regarded as having diagnostic significance with respect to the parent flock, but negative reactions in chick serum samples must be regarded with reserve.

Prior to the initiation of the present study, PPLO had been isolated on three separate occasions from infected embryos from the original antibiotic treated breeder flock. Despite repeated attempts at PPLO isolation during these experiments, no recovery of the organism has been effected.

DISCUSSION

In the light of the observations and data presently available, we believe that certain generalizations regarding the PPLO serum plate test are justified. The sensitivity and specificity of this test have been confirmed and extended by quantitative observations based on serial bleedings of individual dams and their progeny. In order to make the test fully effective, however, one must take into account the variables that can alter its outcome. The use of clean glassware, clear serums and a standarized antigen is certainly basic to the performance of the test. However, other considerations have also been shown to be important; namely, length of reaction time before reading, refrigeration of serum samples before testing, and reproducibility of positive serum reactions.

In the presence of a standard amount of antigen, as the amount of antibody decreases, the size of the aggregates also decreases, and the time before agglutination takes place increases. Therefore, while it is true that strong positive reactions can be read within two minutes, weakly positive serums require a longer reading time. Our experience has shown that the weak reactions will usually be detectable within the 5 minute interval. In attempts to assay the progeny of treated flocks for PPLO reactors, it is important to pick out the birds with agglutinins at the earliest possible time. We have found that a 5 minute reading time interval will allow

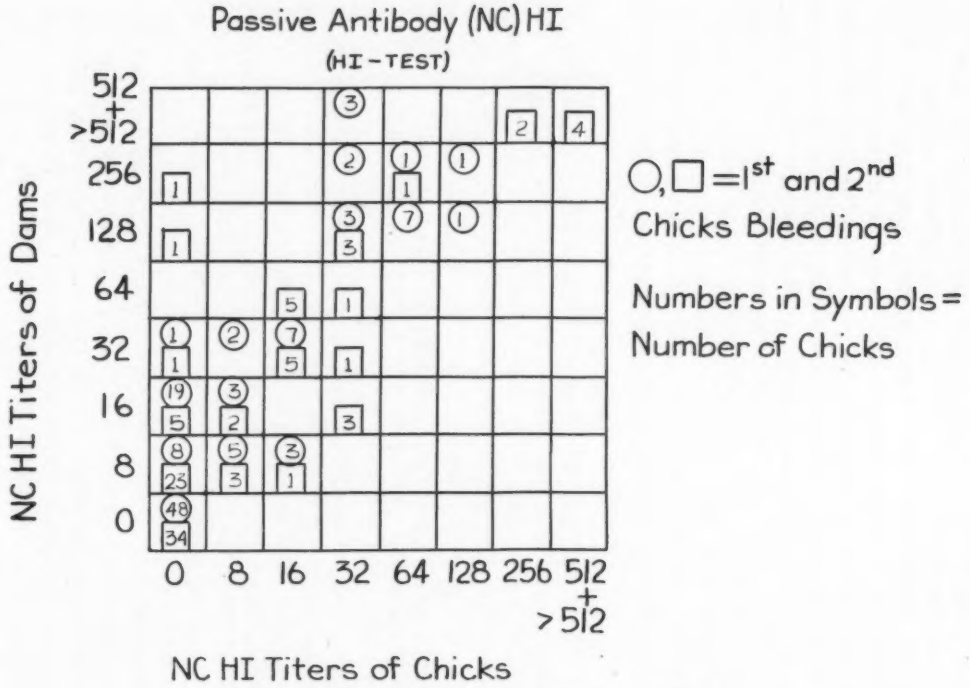

Fig. 4. Newcastle HI titers of chicks and dams.

one to select those weak positive reactors months before the serum from the same bird will react within 2 minutes. This, in effect, increases the sensitivity and hence the effectiveness of the test. Thus, the serum plate test was found to be a highly useful tool in detecting PPLO reactors early in the course of the infection. As a matter of record, positive PPLO agglutinins were observed in the first bleeding of the progeny, and they have persisted for at least 240 days in many individual birds in the absence of discernible clinical manifestations of PPLO or CRD.

Samples that have been drawn, held at room temperature and tested the same day often give erroneous "negative" readings on testing. This may be caused by the presence of complement or other non-specific factors that inhibit the agglutination reaction. Identical split sample testing has demonstrated that holding of serums for a minimum of 24 hours at 4 C before testing avoids this non-specific interference of the agglutination reaction.

Reproducibility of results is an important check on the subjectivity inherent in any system dependent upon human interpretation. One must be able, not only to get the same reaction on retest of the identical serum samples, but to confirm the previous reading by subsequent sampling of the same bird within a short span of time.

In view of Adler's[2] recent report on isolations of two distinct antigenic types of PPLO in chickens, it would appear that control programs at this time will still have to concentrate on the eradication of the type I, or pathogenic, strains of PPLO, and the type II, or non-pathogenic strains, will be the research worker's problem. However, if type II PPLO strains can become invasive under conditions of stress to the birds, then our problem of PPLO control will certainly become more complex; and, if subsequent studies indicate the existence of two antigenic PPLO types in CRD, the use of a polyvalent antigen will be a necessity.

SUMMARY

Use of the serum plate tests for avian PPLO is described

with emphasis on variables which may influence final results of the testing. Detailed serologic data from field experiments on individual dams and their chicks are given in reference to the sensitivity and specificity of the serum plate test, contact transmission from asymptomatic birds, and passive antibody transfer from dam to chick.

In our experience, the use of the serum plate test has been relatively simple, rapid, sensitive and specific in detecting the presence of PPLO antibody in chickens. Its use is indicated in attempts to obtain PPLO clean breeder flocks.

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A TISSUE CULTURE-MODIFIED NEWCASTLE DISEASE VIRUS
II. IMMUNOGENICITY OF THE LIVE TISSUE CULTURE-MODIFIED NEWCASTLE DISEASE VIRUS IN CHICKENS

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THE TISSUE culture-attenuated strain of Newcastle disease virus (NDV) used in this report was described in the first paper of this series.[1] The purpose of the study described herein was to determine the immunogenicity and characteristics of the strain following inoculation into chicks during the first 5 weeks of age.

MATERIALS AND METHODS

Vaccine. The inoculum consisted of the live tissue culture-modified virus (TC) harvested as a supernatant fluid of the culture. Before use, the infectious material was titrated in 8 or 9-day old chicken embryonating eggs, and the suspension was diluted to the desired concentration with phosphate-buffered saline solution (pH 7.2). Penicillin and streptomycin (2,000 units and 1,000 micrograms respectively per 100 ml) were added to the vaccine.

Serology and challenge virus. The technique and

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methods for conducting and expressing the hemagglutination-inhibition (HI) and neutralization (SN) tests were described in the previous paper.[1]

Strain GB Texas 1948 NDV was used for all challenges. The method of preparation and administration has been described.[1]

RESULTS

Experiment 1. Immunological response of 30-day-old chickens to graded doses of TC. Thirty birds, obtained as day-old chicks from a commercial hatchery, were held in isolation quarters until 30 days of age. They were then divided into five groups of six birds each and bled for the HI test. No HI antibodies were detected in any of the birds. The four groups were inoculated intramuscularly with varying dilutions of the virus, the birds in the fifth group serving as controls. The four levels of inoculum consisted of 0.25 ml of TC diluted to contain 1, 100, 10,000 and 1,000,000 embryo mld of virus.

No symptoms developed in any of the vaccinated chicks. The results shown in table I indicated that chicks receiving 1,000,000 or 10,000 embryo mld of the virus developed an HI geometric mean titer (GMT) of 70 and 80 respectively and as SN titer of 10^5 neutralizing doses when the birds reached 7 weeks of age. The chickens which received 1 or 100 mld of virus did not respond as well. Retesting of the birds receiving the two higher levels of the virus at 11 and 16 weeks of age (7 and 12 weeks after vaccination) showed a progressive drop in HI antibody titers. Although the HI titers in these groups dropped below a significant level by the 16th week of age, the SN antibody levels in birds picked at random appeared to remain significantly high. A satisfactory immunity was shown by the fact that the birds resisted intramuscular challenge at these times. The birds of the two groups which received 100 and 1 mld of the virus respectively showed a weaker HI and SN response three weeks after vaccination than the groups which received larger doses of the virus. The duration of significant antibody levels were also shorter

TABLE I

Immunological response of six groups of six chickens each to various embryo lethal doses of tissue culture virus given in 0.25 ml amounts intramuscularly.

No. birds in group	Embryo titer of inoculum	HI titer before vaccination	7 weeks of age		11 weeks of age			16 weeks of age		
			HI GMT[1]	SN[2]	HI GMT[1]	SN[2]	Chal.[3]	HI GMT[1]	SN[2]	Chal.[3]
3	10^6	Neg	70.	10^5	33.	10^3	0/3	....	....	....
3				10^5		10^3	....	16.	10^4	0/3
3	10^4	Neg	80.	10^5	28.	10^2	0/3	12.3	10^4	0/3
3				10^5		10^2	....	....	....	....
3	10^2	Neg	40.	10^5	17.	$<10^3$	0/3	....	$<10^1$	....
3				$<10^4$		$<10^1$	....	Neg	$<10^1$	2/3
3	1	Neg	12.3	10^3	Neg	10^1	3/3	Neg	....	....
3				$<10^1$		$<10^1$	....	Neg	$<10^1$	3/3
3	None (Controls)	Neg	Neg	$<10^1$	Neg	$<10^1$	3/3	....	....	....
3				$<10^1$		$<10^1$	....	Neg	$<10^1$	3/3

[1]Geometric mean hemagglutination inhibition titers determined from HI of individuals in group.
[2]Neutralization titer of pool of equal volumes of 3 sera.
[3]200,000 embryo mld of strain GB given intramuscularly. Results expressed as no. susceptible/no. challenged.

as the dosage of virus was decreased. The absence or presence of low antibody titers was substantiated by the susceptibility of the birds to intramuscular challenge with virulent NDV.

It is evident from this experiment that the immunological response and duration of immunity were dependent upon dosage, and that at least 10^4 embryo mld of the virus were necessary to produce a more durable immunological response with regularity.

Experiment 2. Vaccination of chickens at various ages. To determine the immunogencity of the TC vaccine when given to chicks at various ages, 220 one-day-old chicks were obtained from the hatchery of the California State Poultry Improvement Commission. These birds were a mixture of chicks hatched from 25 breeder flocks and represented offspring of dams of varying immunological status.

When the birds were 5 days old, 81 chicks were bled for serological study, and the flock was divided into four groups as follows: Group I consisted of 110 chicks, all of which were vaccinated at 5 days of age. Group II contained 38 birds picked at random from group I when the latter reached 6 weeks of age. This group was placed in a clean pen and revaccinated. Group III consisted of 60 chicks which were held in isolation for three weeks before being vaccinated. Group IV consisted of 50 chicks which were held as controls. The latter group was placed between groups I and III to determine the degree of spread of the virus within the house. The chicks used for challenge came from this group.

The four groups were kept in a long brooder house which was divided into pens by board partitions extending about 2 feet above the floor, and the wall was continued to the rafters with chicken wire. The floors of the building were of concrete, and the litter consisted of 2 to 3 inches of wood shavings. No precautions were taken by the attendant in feeding and watering the principals to prevent cross-infection.

All of the vaccinated birds were given intramuscularly, 0.25 ml of the TC, which had previously been titrated and diluted to contain 10^4 embryo mld of virus.

TABLE II

Immune response of 3 groups of chicks given vaccine IM either once at 5 days of age, at 5 days and again at 6 weeks of age, or once at 3 weeks of age

Group and vaccination Program	No. birds vacc.	5 days of age		3 weeks of age		6 weeks of age				8 weeks of age				12 weeks of age			
		No. sera	HI GMT	No. sera	HI GMT	No. sera	HI GMT	Challenge IM[1]	Challenge AB[2]	No. sera	HI GMT	Challenge IM[1]	Challenge AB[2,5]	No. sera	HI GMT	Challenge IM[1]	Challenge AB[2,5]
I 5 days	110			....	....		10.7	3/4	0/7[3]	19	10.7	3/10	0/10	19	10.7	2/10	0/9
						88											
II 5 days and 5 weeks	38			....	....					18	28.3	0/9	0/9	20	12.7	2/10	0/10
		81	11.7														
III 3 weeks	60			30	Neg					28	30.3	0/14	0/14	32	25.6	0/16	0/16
IV Controls No vacc.	50			....	....	10	Neg	4/4	6/6[4]	17	Neg	8/8	0/9	15	Neg	8/8	0/7

[1]Challenged IM with 200,000 embryo mld of strain GB Newcastle disease virus. No. susceptible/no. challenged.
[2]Challenged with DeVilbiss atomizer (see text).
[3]All birds had rales by 5th day which subsided and disappeared by 12th day. No nervous symptoms or significant depression.
[4]All birds had rales by 5th day with severe nervous symptoms. One bird died on 12th day.
[5]None of the birds developed symptoms due to defective apparatus.

As shown in table II, the HI GMT of 81 sera, constituting approximately 35 percent of the entire group prior to vaccination, was 11.7 or greater. Of the 81 sera tested, 43 or 58 percent of the sera were negative, whereas the HI titers of 38 sera were more or less equally distributed and ranged from titers of 1:10 to 1:320 or greater. None of the birds given the virus showed any evidence of Newcastle disease involving either the respiratory or the central nervous system.

Immunity to one dose of vaccine at 5 days of age. When the 110 chicks vaccinated at 5 days of age were 6 weeks old, 88 of the birds were bled and, as shown in table II, they had a HI GMT of 10.7. The blood sera from 76 birds showed no HI titer, whereas the remaining 12 showed a titer of 1:10 to 1:80. Four birds were picked at random from each of groups I and IV and were challenged intramuscularly with 200,000 embryo mld of strain GB virus. Three of the four chicks of group I and all four of the control birds from group IV were susceptible. Typical symptoms of clonic spasms and paralysis, resulting in death approximately 24 hours later, were observed. Seven birds from group I and six birds from the control group (IV) were exposed to 10,000,000 embryo mld of the GB NDV strain by the air-borne route by inserting the beak into a tube of a No. 40 DeVilbiss atomizer. The bulb was squeezed 10 times over a period of one minute. Although none of the vaccinated birds died as a result of this exposure, by the fifth day all the chicks had audible rales which gradually subsided and disappeared by the twelfth day. Birds from the susceptible control group (IV), however, were all susceptible to air-borne exposure. Rales were distinctly audible by the fifth day, and paralysis, depression, or hyperexcitability were observed during the following 3 or 4 days. One bird died with paralytic sypmtoms on the ninth day. The remaining birds all showed a residual paralysis and were killed on the twelfth day after exposure.

Tests on a representative number of birds picked at random at 8 and 12 weeks of age, following one intramuscular exposure at 5 days of age, showed very little change in the results that were obtained at 6 weeks. The HI GMT of blood

sera were 10.7 at 8 weeks and 10.7 at 12 weeks. Intramuscular challenge of the chicks at these times showed that 70 percent and 80 percent of the birds respectively, were refractory. Challenge of birds at 8 and 12 weeks by the air-borne route of inoculation proved unsatisfactory. No respiratory or nervous symptoms in any of the birds, including the controls, resulted from this exposure. The failure of this exposure was due to an unnoticed partial obstruction in one of the tubes of the atomizer at the time of its operation.

Immunity to vaccine given at 5 days and repeated 40 days later. Group II consisted of 55 6-week-old birds picked at random from group I. These were placed in clean quarters and revaccinated with 0.25 ml of the TC given intramuscularly. Two weeks after the second vaccination, 18 birds were bled for the HI test. The titers varied from 0 to 1:160 with an HI GMT of 28.3. Nine birds challenged intramuscularly with 200,000 embryo mld of strain GB were all refractory, while the control birds were all susceptible to the virus. The test for immunity was repeated when the birds were 12 weeks old. Although HI titer dropped to a GMT of 12.7 in 20 serum samples tested, 80 percent of the birds were refractory to an intramuscular challenge. The two susceptible birds showed only a transitory ataxia.

The air-borne route of challenge was unsatisfactory in groups I and II challenged at 8 to 12 weeks of age because of the previously described obstruction in the atomizer.

Immunity to vaccine given once at 3 weeks of age. The best immunological response was obtained in the chicks of group III which received one intramuscular dose of vaccine at 3 weeks of age. Chicks with no demonstrable HI antibodies prior to vaccination developed a GMT of 30.3 or greater at 8 weeks of age which decreased to 25.6 at 12 weeks of age. The 14 birds challenged intramuscularly at 8 weeks of age and 16 birds challenged at 12 weeks of age were resistant, whereas the respective control birds from group IV (8 control birds used for each age group) were susceptible.

Experiment 3. Vaccination of 5-day-old chicks by various routes. Because of the irregularity of the immune res-

ponse of 5-day-old chicks obtained from various sources to intramuscular injection of the TC (experiment 2), an experiment was devised using chicks hatched from flocks of known immunological status to the disease. The chicks were divided into groups to determine the relative effectiveness of administering TC by various routes to susceptible and passively immune chicks.

One group of 120 chicks were obtained from the University Poultry Husbandry breeding flock, known to be free of Newcastle disease and not vaccinated against this infection. Another 120 chicks were obtained from a commercial flock known to use an immunization program to insure chicks with a high passive immunity. At 5 days of age, each group was divided into four groups of 30 birds each and wing-banded for identification. Each group was placed in a separate pen in the same poultryhouse. These pens were separated by chicken wire partitions. One group (A) was vaccinated by the intramuscular route, another (B) by the drinking-water method, the third (C) by the conjunctival and intranasal route; the fourth group (D) served as the uninoculated controls.

Methods of administration. Intramuscular inoculations consisted of injecting into the breast muscle 0.25 ml of the TC diluted with buffered saline to contain 10^4 embryo mld of virus per ml (group A). The drinking-water vaccine was prepared by diluting TC to a titer of 10^4 with distilled water containing 16 percent condensed milk. One liter of the vaccine was equally divided between two all-glass drinking receptacles, which were placed before the group for 24 hours (group B). The same preparation used for the intramuscular inoculation was used to instill one drop of the suspension into the conjunctiva of the eye and one drop into the nares of each bird (group C). A commercial intranasal vaccine applicator was used.

Prior to vaccination no detectable SN or HI antibodies were detected in the sera of 17 chickens of the susceptible group bled at random. However, the chicks obtained from dams of the immunized breeding flock presented a HI GMT of 37 and an SN titer of 10^5 (figure 1).

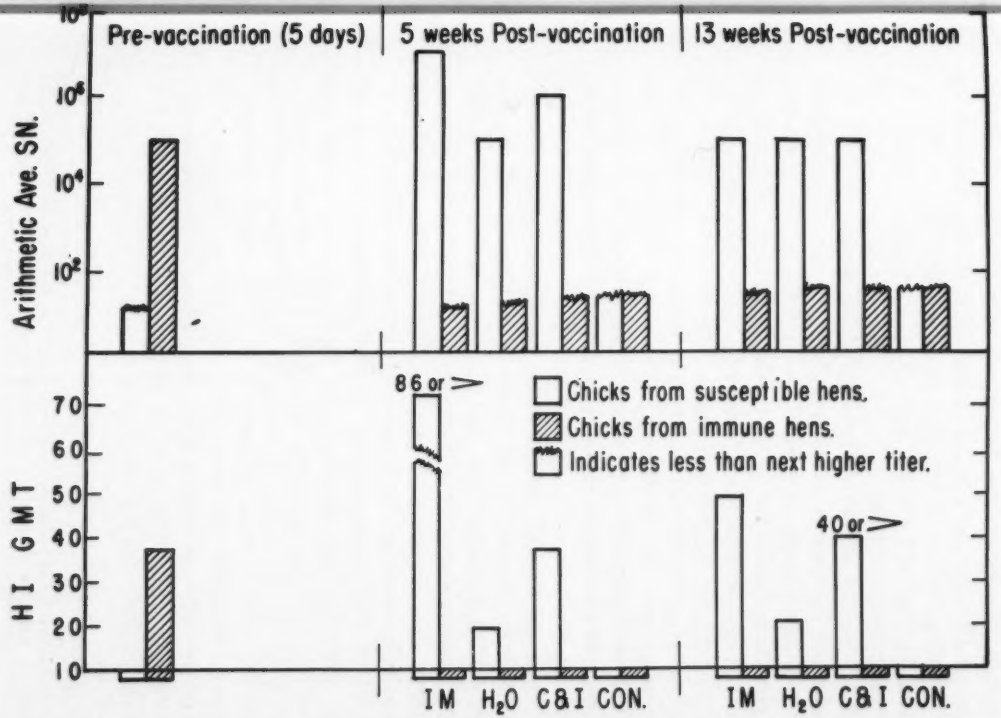

Fig. 1 Serological response of 5-day-old chicks hatched from susceptible and immune hens to tissue culture administered by various routes. IM = intramuscular route; H_2O = drinking water route; C & I = conjunctival-intranasal route; CON = controls; SN = neutralization titers determined on one pool composed of equal volume of sera from each bird collected at time of challenge —see table 3.

No respiratory or nervous symptoms of ND were found in any of the birds during the 13-week observation period. Only five chicks were lost through smothering during the experiment; however, the decreased number of birds in the groups at 13 weeks of age, as shown in table III, was due to loss of wing bands. Since proper identification of the individual birds could not be made, they were not included in the experimental results.

Response of chicks from susceptible dams. Chicks obtained from susceptible dams and given the virus intramuscularly at 5 days of age developed an HI GMT of 86 or greater and an average SN titer of 10^7 by the fifth week. These titers decreased by the 13th week to 49 and 10^5 respectively. Chicks from susceptible dams when exposed to the virus in drinking water or given conjunctival and intranasal inoculations did not develop as good an HI and SN antibody response (figure 1) .The serological response to intranasal and conjunctival exposure was greater than to exposure by the drinking-water method.

These serological results were substantiated by the degree of refractoriness of the birds in each group when challenged by the intramuscular and air-borne routes (table III). All of the birds given the vaccine intramuscularly were refractory to a challenge dose of virulent Newcastle disease virus administered by the intramuscular and air-borne routes.*

All of the nonvaccinated control chickens (group D) which were in close proximity to the vaccinated birds were susceptible to both routes of challenge. The percentage of refractory birds was higher in group C (conjunctival and intranasal vaccination) than in group B (vaccinated by the drinking-water method). In most instances the vaccinated birds showed a greater resistance to air-borne than to intramuscular challenge, whereas the controls appeared to be equally susceptible to challenge by either route.

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* All vaccinated and nonvaccinated birds challenged by the air-borne route at 5 and 13 weeks of age displayed symptoms of moist rales on the third day; the signs disappeared by the eighth day in birds showing no nervous system (NS) involvement. Birds that showed any form of NS involvement or died were considered susceptible.

TABLE III

Results of challenging chicks at 5 weeks and 13 weeks of age following exposure to TC attenuated strain of ND virus administered IM, in the drinking water and intranasally and intraocularly at 5 days of age.

Group	Route of Admin.	Source of Chicks	Challenge Results[1]				
				5 Weeks of Age		13 Weeks of Age	
			Route[2]	no. susc./ no. chal.	% Immune	no. susc./ no. chal.	% Immune
A	0.25 ml. Intra-musc.	Suscept. Dams	IM	0/7	100	0/4	100
			AB	0/7	100	0/4	100
		Immune Dams	IM	7/7	0	5/7	29
			AB	1/7	86	1/5	80
B	Drinking Water	Suscept. Dams	IM	6/7	14	3/5	40
			AB	2/7	71	1/6	83
		Immune Dams	IM	7/7	0	5/7	29
			AB	2/7	71	5/7	29
C	Conjunc-tiva and Intra-nas.	Suscept. Dams	IM	2/7	71	1/7	86
			AB	0/7	100	0/7	100
		Immune Dams	IM	3/7	57	3/4	25
			AB	3/7	57	1/7	86
D	Non Vacc. Control	Suscept. Dams	IM	7/7	0	6/6	0
			AB	7/7	0	....	....
		Immune Dams	IM	7/7	0	8/8	0
			AB	7/7	0	7/7	0

[1]Each bird inoculated intramuscularly with 200,000 mld of strain GB NDV
[2]IM = intramuscular, AB = air-borne route.

Response of chickens obtained from immune dams. Figure 1 shows that none of the sera from chicks hatched from immunized hens contained HI or SN antibodies when the birds reached 5 and 13 weeks of age. Although no antibody response was detectable, some of the birds vaccinated by each method were immune to challenge by intramuscular and air-borne routes. As with chicks from susceptible dams, the vaccinated birds from immune dams also showed a greater resistance to air-borne than to intramuscular challenge (table III).

Although in susceptible chicks the conjunctival and intranasal route of inoculation produced a better immune response (serological or by challenge) than the drinking-water method, the advantage of one route over the other was not demonstrated in the chicks from immune dams.

DISCUSSION

Two desirable characteristics of the TC virus noted in a previous paper[1] were confirmed by the data of these experiments. None of the birds inoculated with the TC virus even as early as 5 days of age showed any clinical manifestations of Newcastle disease or any other untoward effects. No spread of the virus was observed when vaccinated groups of chickens were placed on either side of a group of nonvaccinated controls and separated only by a chicken-wire partition (experiments 2 and 3). This failure of the virus to spread was also observed in other studies in which unvaccinated birds were kept in the same pen with those vaccinated.[2]

The dosage of the TC virus administered to a chicken was found to be an important factor in the development of a satisfactory immunological response. The amount of virus given markedly influenced not only the degree of serological response but also the duration of immunity. The importance of dosage was also observed with other modified living-virus vaccines as well as with a Newcastle disease virus of low pathogenicity used as a vaccine.[3,4,5,6] It was observed in experiment 1 that 0.25 ml of TC with a titer of 10^4 embryo mld was necessary to produce a satisfactory immunological response.

The data indicate that one dose of TC administered intra-

muscularly into 5-day-old chicks from susceptible dams resulted in a substantial serological response, and all birds were solidly immune to an intramuscular challenge of virulent ND virus when they reached 13 weeks of age. Progeny from immune dams in which HI and SN antibodies were demonstrated at the time of vaccination acquired an irregular and less effective immunity. The influence of passive immunity on vaccination of young chicks with the modified virus was corroborated in experiment 2. Chicks from immune dams which were held in isolation for a period of 3 weeks prior to vaccination responded well serologically and were solidly immune to intramuscular challenge when they reached 12 weeks of age.

The routes of administration of TC explored in these experiments indicated that the intramuscular route was superior to the drinking-water or the conjunctival and intranasal routes. The two latter methods of exposure produced a relatively poor and irregular immunological response (table III). This might indicate that the respiratory tract is not very susceptible to the TC virus. The poor immunological response to respiratory tract exposure with TC virus and the consistent failure to isolate TC virus from tissues of vaccinated birds may account for the failure of the virus to spread between vaccinated and susceptible birds.

It was noted in experiment 3 that all groups of vaccinated birds, regardless of the route of administration, were more resistant to air-borne than to intramuscular challenge. No such difference in susceptibility to challenge was noted in the unvaccinated controls. Although the difference in susceptibility was not related to the serum antibody levels (HI and SN) it may be an indication that a measurable degree of immunity was induced in the respiratory tract in TC-vaccinated birds.

The HI response in susceptible chicks receiving one intramuscular dose of TC virus appeared to be intermediate between the responses usually obtained with the B_1 and wing-web Newcastle disease vaccines. Not only did a higher percentage of the TC-vaccinated birds develop HI antibodies, but the titers were usually higher than those obtained with the B_1

vaccine. Of the chicks in these experiments, 58 percent of the sera had titers of 1:40 through 1:320 or greater, 22 percent had titers of 1:10 to 1:20 and only 20 percent of the sera contained no detectable antibodies.

SUMMARY

1. A tissue culture-modified live Newcastle disease virus (TC) administered intramuscularly into 5 and 30-day-old chicks did not produce any manifestations of Newcastle disease or any other untoward reactions.

2. The virus did not spread from vaccinated to susceptible control chickens separated by wire partitions.

3. Chicks lacking passive immunity were solidly immunized for at least 13 weeks by a single intramuscular dose of the vaccine. In contrast, a poor immunological response was obtained when the virus was administered in the drinking water or by instillation into the conjunctival and intranasal routes.

4. The presence of passive immune bodies markedly influenced immunological response to the virus.

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FURTHER STUDIES OF CHICKEN BLOOD TESTS AND THEIR APPLICATION

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A PREVIOUS report[2] in investigations of chicken hematology dealt with normal blood levels in the broiler-type chicken one to ten weeks of age. In this earlier report the blood tests employed and the techniques for carrying them out were described. Since the present communication deals with the same tests and techniques, the discussion concerning them is not reiterated.

It was felt that an investigation of broiler hematology had pertinent value because no comprehensive blood studies on this type of bird has been reported. It should be kept in mind that previous reports on chicken hematology cannot be considered applicable to today's broiler chicken. The stresses of accelerated growth rate resulting from improved breeding and feeding programs have noticeably altered the physiology of today's broiler as compared with its predecessor of the previous decade.

The first phase of the present investigation was to make further studies of the blood of broilers and to fix the normal values for ten blood tests. The work described previously was

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*This work was completed under the Fort Halifax Poultry Disease Fellowship while the senior author was at the University of Maine. This is a portion taken from an M.S. thesis by T. N. Fredrickson.

incorporated into the data obtained in later investigations. The next step in the investigation was to find out how the blood reacted to different environmental conditions, feeds supplemented with antibiotics[1] and blood withdrawal. The final phase of the investigation was to evaluate the blood tests previously carried out in normal birds for the purpose of aiding in the diagnosis of Newcastle disease. The work dealing with normal chickens and those infected with Newcastle disease is herein reported.

MATERIALS AND METHODS

Approximately 400 blood samples were examined to establish normal levels for ten blood tests. Of the birds from which the blood was drawn all but 81 were necropsied to ensure that no lesions caused by disease were present. In the experiment evaluating hematology as a diagnostics aid, 48 broilers, 12 weeks of age, were infected with Newcastle disease virus*. Hemagglutination-inhibition (HI) tests were run on serum samples of birds randomly selected from Newcastle disease susceptible birds. No immunity against the disease was indicated. Marked respiratory symptoms were noticed 3 days after infection and continued to be apparent throughout the period when blood samples were drawn. The Newcastle disease birds were necropsied after blood had been drawn from them. Hemoglobin and microcapillary hematocrit and buffy coat determinations were not made on the blood from the diseased birds.

Accurate hemoglobin readings were considered difficult to obtain from chicken blood. The microcapillary method of hematocrit determination was considered to be preferable to the Wintrobe method since the former entailed less time and equipment. Similar hematocrit readings had been obtained from both techniques. Despite the superiority of the microcapillary method, the Wintrobe was chosen as a substitute becuase it determined, in addition, the erythrocyte sedimentation rate (E.S.R.).

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*5 ml or 1,000 doses of Lederle's B_1 strain of live Newcastle disease virus were administered by the spray method of vaccination.

RESULTS

Tables 1 and 2 give what are considered normal values for broilers, one day to 10 weeks of age, for the following blood tests: Erythrocyte count (RBC), hemoglobin (HB), buffy coat and hematocrit (HT), by the Wintrobe and microcapillary methods; blood and plasma specific gravities, erythrocyte sedimentation rate (ESR), minimal and maximal points of erythrocyte resistance to hemolysis (E. Fragility), total leukocyte count (WBC), and leukocyte differential count. Correlation coefficients were found between several blood tests. These coefficients are given in table 3. The results of blood tests on chickens with Newcastle disease are given in tables 4 and 5.

DISCUSSION

From tables 1 and 2, it is evident that the blood of chickens undergoes considerable alterations in composition during the first two weeks after hatching. The low RBC at this time, especially just after hatching, produces the picture of a normoblastic anemia. Stained smears of young chick's blood reveal many normoblasts and heterochromatic normoblasts in the peripheral blood. The initial WBC is comparatively low due, chiefly, to a low total number of small lymphocytes at this age. Immediately after hatching the total number of heterophils is about that seen in birds 2 weeks of age and older. The myelocytes (most of which are heterophil myelocytes) and the eosinophils are much more numerous than in older birds. At one week of age the WBC count had decreased slightly, due to the reduction of heterophils, eosinophils and myelocytes and despite a doubling by this time of the initial number of small lymphocytes. More or less normal differential counts were observed by the second week after hatching, although the total number of monocytes was somewhat low until the fourth week after hatching. The number of large lymphocytes remains at a uniform level throughout the whole ten weeks of the broilers' life, while the number of other leukocytes fluctuates.

The highly significant correlation coefficients given for three experiments in table 3 indicate that some of the more

TABLE I

The weighted means found for some component levels of the blood of White Rock chickens one to ten weeks of age

Age	RBC millions /mm³	Hb gm/100 ml	Buffy coat cc/100 ml micro	Buffy coat cc/100 ml Wintrobe	Hematocrit cc/100 ml micro	Hematocrit cc/100 ml Wintrobe	Blood SG	Plasma SG	E.S.R. mm/hr.	E. Fragility max. % NaCl	E. Fragility min. % NaCl
1-3 days	1.71	—	.9	—	25.4	—	—	—	—	.19	.43
1 week	1.89	7.30	—	.7	22.5	24.7	1.036	1.015	2.4	.26	.41
2 weeks	2.10	7.28	.9	.8	24.7	27.3	1.039	1.016	1.6	.31	.43
3 weeks	2.13	9.68	1.1	.8	25.0	27.3	1.040	1.017	1.7	.35	.45
4 weeks	2.15	7.20	.9	.9	24.9	27.2	1.041	1.017	1.6	.29	.42
5 weeks	2.11	7.22	.9	.9	26.1	28.3	1.042	1.017	1.5	.30	.43
6 weeks	2.05	7.03	.9	1.1	24.4	26.6	1.043	1.019	1.3	.28	.47
7 weeks	2.32	7.08	.7	.9	27.6	28.7	1.044	1.018	1.3	.28	.41
8 weeks	2.21	7.10	.8	.7	25.6	27.6	1.043	1.018	1.3	.28	.39
9 weeks	2.17	7.40	.8	.8	24.6	27.2	1.042	1.018	1.5	.28	.39
10 weeks	2.18	8.10	.9	.9	25.0	27.3	1.042	1.018	1.5	.30	.41
Total Mean	2.11	7.40	.8	.8	25.1	27.2	1.041	1.017	1.5	.28	.42
No. of chickens	419	185	138	423	176	423	423	410	423	415	417

SG = specific gravity. RBC = red blood cells. E.S.G. = erythrocyte specific gravity.
E. fragility = erythrocyte fragility.

TABLE II

WBC differential values in percent of total count and actual number /mm³ for 379 White Rock chickens

Age in weeks	Small Lympho-cytes	Large Lympho-cytes	Mono-cytes	Myelo-cytes	Eosino-phils	Baso-phils	Hetero-phils	WBC Thousands /mm³
0	27*	22	3	7	6	6	29	16,000
	4,320	3,520	480	1,120	960	960	4,640	
1	52	24	3	1	1	3	16	14,100
	7,332	3,384	423	141	141	423	2,256	
2	51	23	2	1	1	2	20	19,700
	10,047	4,531	394	197	197	394	3,940	
3	49	21	3	1	1	3	22	18,000
	8,820	3,780	540	180	180	540	3,960	
4	60	14	4	1	1	3	17	28,600
	17,160	4,004	1,144	286	286	858	4,862	
5	53	13	6	1	1	3	23	27,200
	14,416	3,536	1,632	272	272	816	6,256	
6	56	12	5	1	1	3	22	28,500
	15,960	3,420	1,425	285	285	855	6,270	
7	55	11	6	1	1	4	22	29,500
	16,225	3,245	1,770	295	295	1,180	6,490	
8	53	12	6	1	1	3	24	24,400
	12,932	2,928	1,464	244	244	732	5,856	
9	53	14	4	1	1	1	26	24,300
	12,879	3,402	972	243	243	243	6,318	
10	51	14	5	1	1	1	27	27,700
	14,127	3,878	1,385	277	277	277	7,479	
Mean	51	16.5	4.5	1	1	3	23	23,455
	11,781	3,812	1,040	231	231	693	5,313	

*Upper figures represent percent of total count.

simple blood tests, which employ a minimum of equipment and technical skill, provide considerable information on the blood components. The whole blood specific gravity is closely interrelated with the RBC count and hemoglobin content of the blood. The hematocrit indicates quite closely the RBC count. The interrelationship of the buffy coat is highly correlated with the WBC count in one experiment but not the other two.

The data in tables 4 and 5 indicate that the WBC fluctuated considerably during Newcastle disease. The most apparent changes in the WBC count were a leukopenia on the eleventh day after infection and a leukocytosis on the fourteenth day. Similar fluctuations in WBC were noted by Machado[3] but at different times after the initial infection with

TABLE III

Interrelationships of some blood tests

Exp. No.	X	Y	r	y	n-2	P.05	P.01
808	Hb	Blood SG	.652		37	.325	.418
808	Ht	RBC	.570	86,400	35	.325	.418
808	Ht	Blood SG	.709	1.0011	37	.325	.418
808	Ht	Hb	.402	.33	35	.325	.418
808	BC	WBC	.122	——	37	.325	.418
1194	Hb	Blood SG	.443		51	.273	.354
1194	Ht	RBC	.820	91,100	48	.273	.354
1194	Ht	Blood SG	.776	1.0012	51	.273	.354
1194	BC	WBC	-.055	——	46	.288	.372
1194	BC	ESR	.014	——	45	.288	.372
F	Ht	RBC	.721	80,800	249	.138	.181
F	Ht	Blood SG	.618	1.0017	243	.138	.181
F	Bld.SG	RBC	.438		249	.138	.181
F	BC	WBC	.503	——	191	.138	.181
F	BC	ESR	.531	——	191	.138	.181

Exp. No. = experiment number or letter.
X = independent variable.
Y = dependent variable.
r = coefficient of correlation.
y = predicted Y from the unit value of.
n-2 = degrees of freedom.
Hb = hemoglobin.
Ht = hematocrit.
BC = buffy coat.
P = probability at the 5% and 1% levels.
SG = specific gravity.

Newcastle disease virus. In this investigation the leukopenia was due to a decrease in the numbers of all classes of leukocytes but the leukocytosis was due to a monocytosis and heterophilia. While the buffy coat more than doubled during the leukocytosis it did not decrease appreciably during the leukopenia. The plasma specific gravity increased during leukocytosis while the E.S.R. was accelerated to the greatest degree during the leukopenia. Other blood tests showed a very moderate state of anemia during both the leukopenia and the leukocytosis.

CONCLUSIONS

The normal values given in table 1 and 2 illustrate the importance of comparing birds of an age group with the normal values for that particular age.

TABLE IV

Some aspects of the blood picture of White Rock chickens before and after artificial infection with Newcastle disease virus

Days from infection	Ht cc/100ml (Wintrobe)	Blood SG	RBC millions	E.S.R. mm/hr.	E. Fragility % NaCl min.	E. Fragility % NaCl max.	Buffy Coat cc/100ml	Plasma SG
6 days before	27.2	1.045 .026*	2.19	1.4	.35	.23	.9	1.019
4 days before	27.5	1.045 .027*	2.26	1.6	.37	.27	1.0	1.018
3 days after	26.2	1.044 .025*	2.13	1.6	.43	.31	.9	1.019
4 days after	27.8	1.044 .025*	2.19	1.5	.41	.28	1.0	1.019
6 days after	25.2	1.044 .024*	2.11	1.8	.43	.28	1.1	1.020
8 days after	26.9	1.044 .025*	2.08	1.6	.41	.29	1.3	1.019
10 days after	27.5	1.045 .026*	2.23	1.8	.43	.28	1.0	1.019
11 days after	26.6	1.043 .024*	1.89	2.3	.44	.31	1.2	1.019
14 days after	25.8	1.045 .023*	2.07	2.0	.40	.29	2.6	1.022
16 days after	28.6	1.044 .024*	2.00	1.2	.40	.29	1.0	1.020

*This figure represents (Blood SG) — (Plasma SG) i.e. 1.045-1.019 = 0.026

TABLE V

The relative and absolute leukocyte differential count in chickens following artificial infection with Newcastle disease virus in White Rock chickens

Days after Infection	WBC thousands /mm³	Small Lympho-cytes	Large Lympho-cytes	Mono-cytes	Myelo-cytes	Eosino-phils	Baso-phils	Hetero-phils
3	16.8	58*	3	7	3	1	2	27
		9,744**	504	1,176	504	168	336	4,536
4	23.6	63	5	8	3	0	1	20
		14,868	1,180	1,888	708	—	236	4,720
6	17.1	69	3	9	3	1	2	15
		11,799	513	1,539	513	171	342	2,565
8	17.7	52	7	10	2	0	1	27
		9,204	1,239	1,770	354	—	177	4,779
10	21.9	52	7	13	2	0	1	26
		11,388	1,533	2,847	438	—	219	5,694
11	12.8	59	4	5	1	0	2	30
		7,552	512	640	128	—	256	3,840
14	38.0	35	2	20	2	1	1	40
		13,300	760	7,600	760	380	380	15,200
16	17.7	54	4	6	1	1	2	32
		9,558	708	1,062	177	177	354	5,664

*percentage **thousands/mm³

The investigation of blood tests applied to diseased chickens indicates that in virus diseases the WBC count is the most important diagnostic blood test, especially when used in conjunction with the leukocyte differential count. The buffy coat, blood and plasma specific gravity and E.S.R. determinations are of considerable value in diagnostic procedures but alterations in these tests are not as apparent quantitatively as in the WBC count. Although the E.S.R. was accelerated during the course of Newcastle disease, the increase was rarely more than 2 mm/hr and usually about 1½ mm/hr. Such numerically small differences between normal blood and that of diseased birds hampers the applicability of the E.S.R. in poultry pathology. It has been observed that the plasma specific gravity increases with the onset of either viral or bacterial diseases. The whole blood specific gravity is increased in such cases but only due to the effect of the plasma constituents. The specific gravity of both blood and plasma should be found in order to gauge whether disease or increased hemoglobin content is responsible for a high blood specific gravity reading. The greatest increase in plasma specific gravity was during the period of leukocytosis.

In disease conditions involving a primary or secondary anemia, the hematocrit and blood plasma specific gravity determinations would be very valuable in making a diagnosis.

SUMMARY

Normal values for 10 different blood tests on broiler chickens one day to ten weeks of age are given. Approximately 419 broilers were examined to find these values which are expressed as weighted means.

In Newcastle disease the WBC and leukocyte differential count were of the greatest diagnostic value. The erythrocyte sedimentation rate, buffy coat values and plasma specific gravity determinations were also altered during the disease.

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THE USE OF ERYTHROMYCIN IN THE TREATMENT OF INFECTIOUS TURKEY SINUSITIS DURING A NATURAL OUTBREAK

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INFECTIOUS turkey sinusitis (ITS) has established itself as a disease of major importance to the turkey industry. Considerable attention has been focused on the problem of eradication and control of this disease. Argyrol and silver nitrate injected directly into the infected sinus accompanied by surgical drainage was used quite extensively in the past to relieve this condition[2,4,17,18,24] although there was no beneficial effect on lower respiratory involvement. Several investigators reported that streptomycin at a level of 100 to 200 mg per sinus had been used successfully in the treatment of ITS.[1,3,7,11,13,16,21,23] Reports are becoming more numerous on the effect of other antibiotics such as oxytetracycline, chlortetracycline and erythromycin in an effort to control this condition.[8,10,12,25,26] Pleuropneumonia-like organisms (PPLO) have been associated with ITS as well as chronic respiratory disease CRD, of chickens.[9,14,15,19] The present report describes the use of erythromycin in the treatment of a naturally occurring outbreak of ITS.

This study was suported by a grant from Eli-Lilly and Co., Indianapolis, Indiana.

Case history: An outbreak of ITS in a flock of 18,000 Broad Breasted Bronze turkey poults in northwestern Ohio was called to our attention. The owner reported that nearly 20 birds were treated with penicillin intramuscularly in April, 1957 with no apparent response. In the ensuing weeks the disease continued to spread. When the flock was visited on June 25, 1957, the turkeys were approximately 14 weeks of age. They were kept in groups of about 3,000 birds on an alfalfa and clover range. About 20 percent of the entire flock was affected with ITS. The clinical signs included sinusitis, usually bilateral, viscid discharge from the nostrils, swelling of the infraorbital sinus, with the eyes almost closed. Some birds exhibited respiratory distress. The diseased birds showed retarded growth with roughened feathers and depression. The mortality rate among the affected birds was nearly 10 percent. Blood samples from a few sick birds were collected and were tested for PPLO antibodies using the hemagglutination inhibition test.[5] All samples were positive at a titer ranging from 1:160 to 1:640 serum dilution. Diagnosis was confirmed by isolating PPLO from the sinus fluid in Morton's medium[20] enriched with 2 percent Difco serum fraction, in phenol red maltose broth[9] and on Difco PPLO agar plates.[12] Biochemical reactions of the isolated agent were studied in 14 carbohydrates. Attempts to reproduce the condition experimentally were successful by the use of one ml of the sinus fluid alone or one ml of PPLO broth culture inoculated into the infraorbital sinus in four 8 to 10-week-old turkeys. These birds were serologically negative for PPLO antibodies at time of exposure. They showed typical symptoms of ITS 14 to 21 days post-inoculation and PPLO were recovered from the sinus fluid of the four experimentally infected birds.

Histopathological examination was made of the sinus, air sac and lung from some untreated naturally infected turkeys. The mucous membrane of the sinus was thickened with marked hyperplasia of the pseudostratified, ciliated, columnar epithelium. In addition, the mucous membrane was thrown into folds and papillary projections, probably due to the thickening and hyperplasia. There was marked lymphoid hyperplasia

in the lamina propria with some infiltration of cells into the mucous membrane. Nearly all capillaries were filled with blood.

The membrane of the posterior air sac was greatly thickened. There was hyperplasia of the layer of ciliated columnar epithelium in many with resulting folding and papillary projections. There were increased numbers of capillaries in the stroma when compared to an unifected membrane.

Interalveolar capillaries throughout the lung section were filled with blood and widely scattered, small, circumscribed abscesses were seen.

Six hundred twenty-five infected turkeys were randomly divided into five groups. They were kept separate from the rest of the flock by a wire fence and were identified by wing bands. Four of the groups were treated and the fifth was left as control. Group 1 consisted of 100 birds injected with 100 mg erythromycin glucoheptonate (Illotycin) into the sinus, while a second group of 175 birds were given only 75 mg of erythromycin-Lilly into the sinus. Group 3 contained 100 turkeys each of which received 50 mg erythromycin glucoheptonate into the sinus. Group 4 was made up of 100 infected birds and received 25 mg of erythromycin pellets implanted subcutaneously in the side of the neck. The remaining 150 infected birds were used as controls. When a large swollen sinus was present, about 5 ml of the sinus fluid was withdrawn before medication. The duration of the experiment was 6 months.

RESULTS

Erythromycin glucoheptonate therapy at a level of 100 mg per sinus was effective in treating ITS. The disease regressed rapidly when the antibiotic was adminstered into the infected sinus. In group 1, 88 percent of the infected birds recovered and the swollen sinuses were completely normal within a period of 4 to 6 weeks postinoculation. The rate of recovery in the birds of group 2, which received 75 mg per sinus, was only 63 percent. Table I shows the effect of various concentration of erythromycin in treatment of ITS. It was noted that the rate of efficiency of the drug was directly pro-

TABLE I

Effect of erythromycin administration in treatment of infectious turkey sinusitis

Antibiotic	No. birds	Dosage[1] mg	Route of inoc.	Recovery	Partial Recovery	No recovery	Died	% recovery
Eryth. gluco-heptonate	100	100	IS[2]	88	7	2	3	88
Eryth. Lilly	175	75	IS	116	21	28	10	63
Eryth. gluco.	100	50	IS	60	22	10	8	60
Ervth. pellets	100	25	SC[3]	12	32	50	8	12
Control	150	0	0	0	2	132	16	0

[1] Dosage of antibiotics per sinus. [2] IS = Intra sinus.
[3] SC = Subcutaneous inoculation per bird.

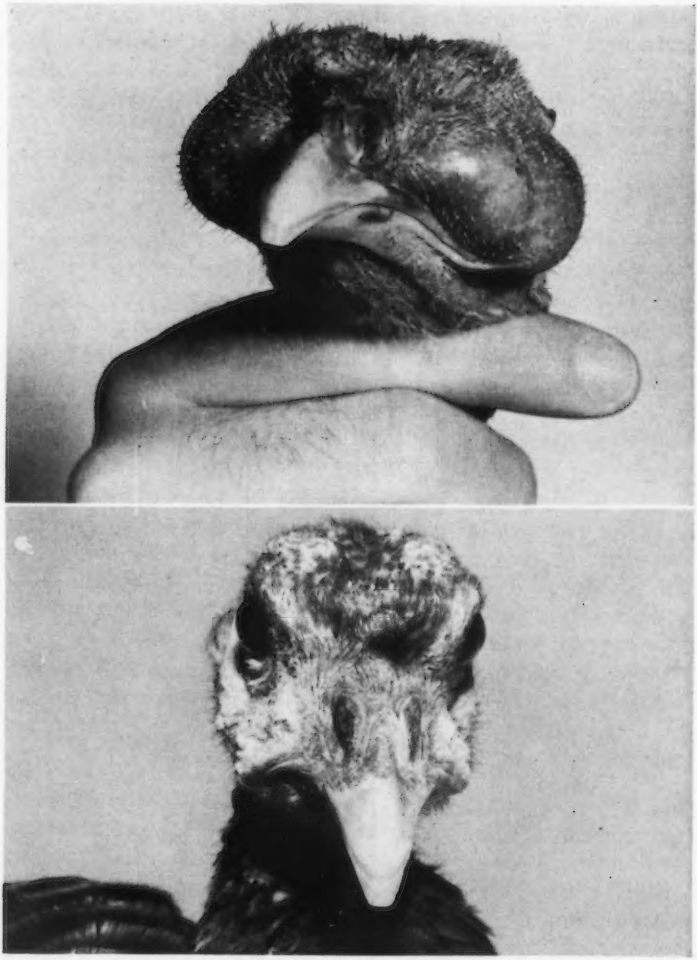

Fig. 1. (top) Turkey naturally infected with bilateral sinusitis before medication.

Fig. 2. (bottom) Same turkey 5 weeks after infection with 100 mg erythromycin per sinus.

portional to the dosage used when the medication was administered directly into the sinus. However, 25 mg pellets of erythromycin per bird showed a low rate of recovery as compared wth the other treated groups. The mortality rate was reduced in the treated group when compared to the untreated control group. The treated recovered birds showed marked improvement in appearance over the non-treated controls.

A study of the biochemical reaction of PPLO in 14 sugars, revealed that this strain produced acid with no gas in glucose, maltose, trehalose and sucrose but did not ferment arabinose, galactose, lactose, levulose, inositol, mannitol, raffinose, rhomnose and xylose as judged by change in color of the indicator and pH measurement. Trials were repeated three times to support the present findings.

DISCUSSION

Erythromycin glucoheptonate was found to be effective in controlling cases of ITS in a field outbreak. Treatment with 100 mg of the antibiotic per sinus proved to be 88 percent effective. The rate of recovery of erythromycin was in direct proportion to the dosage used. It has been reported[12] that 50 mg of the same antibiotic per sinus gave good results in the treatment of experimentally infected turkeys with ITS when treated early in the infection. Under field conditions in this experiment, 50 mg per sinus was much less effective in promoting recovery than 100 mg. The present data are in agreement with the results in other reports.[12,26] Injection of the antibiotic appears to be the preferred route of treatment. The mortality rate was significantly lowered in group 1 and 2 when treated with 100 and 75 mg of the antibiotic respectively. Erythromycin pellets implanted subcutaneously in the neck produced a low rate of recovery but the severity of the infection was decreased. Other investigators[6] reported on a field outbreak of ITS due to strains of PPLO resistant to streptomycin and partially resistant to oxytetracycline and chlortetracycline. It can be concluded that various strains of avian PPLO vary in their susceptibility to the action of different antibiotics and a number of drugs may have to be tried before one is found which will be effective against a given infection.

SUMMARY

A field outbreak of ITS was encountered. The clinical signs of the disease included bilateral sinusitis with respiratory distress. Blood samples from sick birds showed a hemagglutinating titer for PPLO antibodies ranging from 1:160 to 1:640 serum dilution. An avian strain of PPLO was isolated from the sinus fluid of infected birds. The mucous membrane of the sinus was thickened with hyperplasia of pseudostratified, ciliated columnar epithelium. Lymphocytic hyperplasia of the lamina propria was present.

On hundred mg of erythromycin glucoheptonate per sinus in 100 infected birds was 88 percent effective in promoting clinical recovery in 4 to 6 weeks. Concentrations of 50 mg in 100 birds and 75 mg of erythromycin per sinus in 175 birds produced 60 and 63 percent recovery respectively. Erythromycin pellets implanted subcutaneously in 100 birds were 12 percent effective. However, all concentrations reduced the severity of the condition in birds which did not recover and reduced the death losses. Intrasinus injection was the prefered route of administration. The biochemical reaction of an avian strain of PPLO was also studied.

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STUDIES ON A SERUM NEUTRALIZATION TEST FOR THE DIAGNOSIS OF LARYNGOTRACHEITIS IN CHICKENS

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THE serum neutralization test for the diagnosis of infectious laryngotrachetis (LT) in chicks has not been used as a routine diagnostic test in the laboratories of the United States. Burnet[1] developed a plaque counting technique for estimating virus concentration of suspensions and adapted it for use in a serum neutralization (SN) test. Pulsford and Stokes[3] employed a similar procedure for conducting a survey of flock infection by an SN test. Their interpretation of the test was based upon the percentage of reduction in the plaque count as compared to a control. Simmons, *et al.*[5] also make reference to an SN test using Burnet's plaque counting technique.

In recent studies by Hitchner and White[2] on comparative embryo inoculation techniques for determining virus concentrations of LT virus suspensions, it was demonstrated that the dropped chorioallantoic membrane technique consistently gave higher titers than other methods employed. With a titration technique that gave consistent results, it seemed plausible that if serum antibodies were present an SN test could be developed, using serial ten-fold dilutions of known

virus with serum, and calculating the endpoints of infectivity by the method of Reed and Muench.[4]

MATERIALS AND METHODS

The procedure for conducting the LT SN test was patterned after a method in common usage for Newcastle and infectious bronchitis viruses. The virus suspensions were prepared from infected chorioallantoic membranes (CAMs) harvested approximately 5 days postinoculation. The CAMs were weighed, ground in a mortar with sterile sand and suspended in equal parts (w/v) of tryptose broth containing 100 units of penicillin and 1 mg of dihydrostreptomycin per ml. The suspension was centrifuged in an angle centrifuge at 2,000 rpm. for 10 minutes. The supernatant fluid was then diluted 1:5 to make a 10^{-1} dilution of the original CAM suspension. Subsequent ten-fold dilutions were made in 4.5 ml blanks of tryptose broth, or in quantities sufficient to conduct the desired number of SN tests. For subsequent use in SN titrations, the supernatant fluid of the 1:2 dilution was distributed in glass ampules which were sealed by flame and stored in a freezer at —40 C.

Selected ten-fold dilutions of the virus were mixed with equal volumes of serum that had been previously heat-inactivated in a water bath at 56 C for 30 minutes. The virus control was mixed with equal volumes of tryptose broth. The serum-virus mixtures were thoroughly shaken, then held at room temperature for at least one hour prior to embryo inoculation.

Ten or 11-day-old embryonated eggs were used for inoculation by the dropped-membrane technique. A sharpened nail punch was used to drill a hole through the shell over the air cell and on the side of the egg after first swabbing these areas with iodine. In drilling the side hole, it was necessary to use care to avoid going too deep with the drill and puncturing through the CAM. After preparing the holes, the tray of eggs was placed in a darkened room. With the aid of a light for illuminating the egg and a 10 ml capacity rubber bulb, a false air cell was created by evacuating the air from the normal air

cell. The use of the bulb was greatly facilitated by adapting a rubber-sleeved stopper to the tip of the bulb by means of a short piece of glass tubing.

Each of five embryos was inoculated on the CAM of the false air cell with 0.1 ml of each of the selected serum-virus mixtures. The inoculum was delivered with a 1.0 ml tuberculin syringe and needle on the CAM after the tip of the needle had passed the shell. After inoculation, both holes were sealed with melted paraffin and the eggs in the tray were tilted in the opposite direction to more widely distributed the inoculum. The eggs were then returned to the incubator.

The eggs were candled daily to determine embryo mortality. Five to seven days postinoculation, the CAMs were examined for the presence or absence of gray, circular plaques. Any embryo which displayed one or more plaques was considered positive for LT infection. It was frequently observed that the plaques were not visible in dead embryos, although in the same dilution group, the living embryos plainly exhibited good plaques. The opacity of the plaques seemed to disappear rapidly after death of the embryo. Therefore, dead embryos were not considered in the final calculations except those that died on the day of the terminal examination. The embryo infective $dose_{50}$ (EID_{50}) end points were calculated by the method of Reed and Muench.[4]

Two trials were conducted to establish the approximate time required for maximum neutralization in the serum-virus mixtures. A different antiserum pool was used in each trial. The serum-virus mixtures were inoculated into embryonated eggs after holding at room temperature for intervals of 15, 30, 60 and 120 minutes. In the first trial a control from susceptible birds was included, inoculating it at the 15 and 120 minute intervals only. To assure that the time interval involved had no influence on the titer of the virus control, the virus in trial 1 was titrated in embryos immediately before and immediately after inoculating the serum-virus mixture.

Frequently, when small serum samples are submitted, dilution of the samples is necessary to provide adequte amounts to conduct an SN test. To determine if dilution of the

serum sample altered the quantitative specificity of the SN test, six LT positive serums and one negative serum were used in dilutions of 1:2 and 1:5. The EID_{50} titers were corrected to compensate for the dilution factor.

In experimental studies on the SN test with infectious bronchitis virus, it has been observed that pooled serums will often give a higher SN titer than the mean of the individual bird titers. To see if pools of serum gave more favorable SN titers than the individual bird titers, three pools were made using equal quantities of serum from five birds each. After conducting the SN test on each of the bird samples plus the pools, the mean titer of each of the groups of five birds was calculated and compared with the titer of the pool.

Two trials were conducted to determine the postvaccination interval required before serum antibodies were detectable by the LT SN test. The vaccination was performed by application of the virus to the vent of the birds by means of a brush. In trial 1, a strain designated as L-8 was used for vaccination and in trial 2, a strain designated as L-6 was used. Five birds from each of the vaccinated groups were bled at weekly intervals after vaccination for four and five weeks postvaccination, respectively.

To determine the neutralization indices (NI) of LT susceptible birds, SN tests were conducted on serums from 40 known susceptible birds.

RESULTS

The supernatant fluid prepared from virus-infected CAMs provided an inoculum with high concentration which could be held in the frozen state at -40C for at least three months (longest period tested) without a significant loss in titer. Table I shows the titration results over a three month period of pooled CAM harvested on the sixth day postinoculation and held frozen in the 1:2 dilution. When first titrated, the EID_{50} of the freshly prepared suspension was not obtained but exceeded $10^{8.5}$/ml. One month later the titer had dropped approximately one log, but for the remainder of the test period the virus concentration was quite stable.

TABLE I

The stability of a LT virus suspension held at -40 C for a three-month period

Date titrated	EID_{50} log_{10} Titer/ml
	=
4/11/57	>8.5
4/22/57	8.2
5/7/57	7.4
5/10/57	7.5
5/14/57	7.6
5/17/57	7.5
5/21/57	7.4
6/3/57	7.6
6/4/57	7.5
6/11/57	7.6
6/18/57	7.4
6/25/57	7.7
7/1/57	7.2
7/5/57	7.8
7/12/57	7.3

A preliminary trial on the influence of heat inactivation of the serum on the SN titer was conducted on four immune pools and one susceptible pool. The results shown in table II were not conclusive and addtional studies on heat inactivation were done at the time studies were carried out on the incubation period required for complete neutralization. These results are shown in table III, and as in the previous trial, were not conclusive that heat inactivation of the serum had any additional advantages. Because there was the possibility that inactivation of the serum would eliminate nonspecific agglutinins in susceptible serum, it was decided to routinely inactivate all serums before conducting the test.

The results obtained on the study of the incubation interval at room temperature for the serum-virus mixture to attain maximum neutralization are given in table III. In the first trial, neutralization had reached its maximum after 30 minutes' incubation. In addition to the antiserum, a susceptible serum pool and virus control were titrated at the 15 minute and 2 hour intervals. Neither the susceptible serum nor the virus control showed any significant difference in titer at the intervals tested; in fact, the virus control titer was identical,

TABLE II

Heat inactivated vs. non-inactivated serum for the LT SN test

Serum identity	Treatment	EID_{50} log_{10} titer/ml
LT susceptible	Inactivated	1.0
	Not inactivated	1.4
L-5 Antiserum pool	Inactivated	2.4
	Not inactivated	3.2
L-6 Antiserum pool	Inactivated	3.0
	Not inactivated	3.7
L-8 Antiserum pool	Inactivated	2.7
	Not inactivated	3.2
L-9 Antiserum pool	Inactivated	2.8
	Not inactivated	2.6

TABLE III

Influence of incubation time on LT SN titer

Trial No.	Serum Identity	Treatment	EID_{50} log_{10} titer/ml Time of incubation 15′	30′	60′	120′
1	L-6 Antiserum Pool	Inactivated	2.0	3.8	3.3	2.5
		Not. inactiv.	2.5	3.5	3.2	3.3
	LT Susceptible Pool	Inactivated	0			0
		Not inactiv.	0			0.5
2	L-8 Antiserum Pool	Inactivated	2.9	2.5	3.2	3.4
		Not inactiv.	2.2	2.0	3.5	3.2

with an EID_{50} titer of $10^{7.5}$. In the second trial, neutralization of the virus was not complete until after one hours' incubation. On the basis of these results, the serum-virus mixtures in all subsequent SN tests were held at room temperature for at least one hour before the inoculation into embryos.

Results on the study of serum dilution in relation to the SN titer are shown in table IV. They reveal no significant pattern as to the effect of dilution on the serum titer. From the standpoint of a diagnostic test, it appears a 1:2 or 1:5 dilution is equally as accurate in detecting immune birds as when undiluted serum is used.

The SN test run on five individual bird serum samples and on pools of equal quantities of serum from each showed

no great difference between the results obtained on the pooled sample and the calculated mean of the individual samples as shown in table V.

Two trials were conducted to determine the postvaccination interval required for the detection of SN antibodies in the serum. The results are presented in table VI.

From the results of studies on the NI of susceptible serum, shown in table VII, an NI of greater than 10 could be considered as evidence of antigenic response to LT virus. As seen in trial 1 (table VI) antibodies were detectable in three of five birds on the eighth and fourteenth days postvaccination and had apparently reached maximum levels by the third week. In trial 2, using a different strain of virus, all five birds exhibited a positive NI by the seventh day, and four out of five were positive on the fourteenth day postvaccination. Again, in this trial, maximum antibody levels were not attained until the third week.

The results of SN tests on 40 serum samples from LT susceptible birds are given in table VII. From the data pre-

TABLE IV

Serum dilution and its relation to SN titer

Serum sample	Dilution	EID_{50} log_{10} titer/ml
1	undiluted	3.8
immune	1:2	3.4
	1:5	3.2
2	undiluted	3.1
immune	1:2	2.8
	1:5	3.5
3	undiluted	3.0
immune	1:2	3.4
	1:5	3.8
4	undiluted	2.9
immune	1:2	3.1
	1:5	2.9
5	undiluted	3.2
immune	1:2	3.8
	1:5	3.7
6	undiluted	3.7
immune	1:2	3.1
	1:5	2.9
7	undiluted	0.2
susceptible	1:2	0.3
	1:5	0.6

TABLE V

SN tests on five individual LT immune samples and on the pool of equal parts of the corresponding samples

Group no.	Bird no.	EID_{50} log_{10} titer/ml
1	3493	2.7
	4222	3.2
	3487	4.0
	4307	3.7
	3489	3.2
	Mean	3.3
	Pool	3.4
2	4266	3.0
	4186	3.6
	4225	3.2
	4188	2.9
	4190	3.0
	Mean	3.1
	Pool	3.7
3	4270	2.9
	4143	2.5
	4150	3.7
	4250	3.5
	4788	4.0
	Mean	3.3
	Pool	3.7

sented, the SN titer of 99.7 percent ($\overline{X} \pm 3s$) of serums from non-exposed chickens would not be expected to exceed $10^{1.28}$ or 19 neutralizing doses; similarly, the titer of 95.4 percent ($\overline{X} \pm 2s$) of such serums would not be expected to exceed $10^{0.95}$ or 9.3 neutralizing doses.

DISCUSSION

The results of this study on the SN test for LT demonstrated that it can be advantageously used as a diagnostic aid and in immunological studies with LT virus. It appears that the dropped CAM technique is essential for obtaining satisfactory results. The dropped membrane technique of embryo inoculation is highly sensitive in detecting very small quantities of virus and possibly even individual virus particles. With other methods of embryo inoculation, titers have been as much as two logs lower, which would make such inoculation techniques less effective in an SN test in view of the fact that SN levels were found to be in the range of 10^2 or 10^3 logs and rarely exceeded a log titer of 10^4.

TABLE VI

The levels of SN antibodies at intervals postvaccination

Trial 1				Trial 2			
Interval postvac.	Bird no.	EID_{50} log_{10} titer/ml		Interval Postvac.	Bird no.	EID_{50} log_{10} titer/ml	NI*
Contact controls 8 days postvac.	4095	0.7	5		4263	0.0	0
	4096	0.1	1		4191	0.0	0
	4097	0.0	0	0	4224	0.0	0
	4098	0.2	2	Prevac	3492	0.5	3
	4099	0.0	0		4223	0.1	1
8 days	4033	0.9	8	7 days	4108	>2.0	>100
	4035	>1.8	>60		4182	>2.0	>100
	4036	1.8	60		4185	1.3	20
	4037	1.7	50		4271	>2.0	>100
	4039	0.9	8		3494	>2.1	>130
14 days	4053	0.6	4	14 days	1	2.2	160
	4082	0.1	1		2	<1.0	<10
	4083	2.1	125		3	1.2	16
	4085	1.6	40		4	2.2	160
	4089	1.3	20		5	2.0	100
21 days	4088	2.5	320	21 days	3493	2.7	500
	4090	3.2	1585		4222	3.2	1585
	4091	2.6	400		3487	4.0	10,000
	4092	3.0	1000		4307	3.7	5,000
	4093	1.9	80		3489	3.2	1585
28 days	4053	1.9	80	28 days	4266	3.0	1000
	4084	2.2	160		4186	3.6	4000
	4086	2.3	200		4225	3.2	1585
	4087	2.2	160		4188	2.9	800
	4094	3.0	1000		4190	3.0	1000
				35 days	4270	2.9	800
					4143	2.5	315
					4150	3.7	5000
					4250	3.5	3160
					4788	4.0	10,000

*NI = neutralization index = antilog of the EID_{50} titer.

The use of five embryos per dilution is sufficient to give results satisfactory for a diagnosis of LT. Because of the inability to make satisfactory infectivity determinations on the embryos that die during the postinoculation period, it would be advisable in highly critical studies to inoculate seven or

TABLE VII

Results of LT SN Tests on serum from susceptible birds

Sample no.	EID_{50} log_{10} Titer/ml	NI
1	0	0
2	0.2	2
3	0.3	2
4	0	0
5	0.2	2
6	0	0
7	0.5	3
8	0.5	3
9	0	0
10	0.6	4
11	0	0
12	0.5	3
13	0.1	1
14	0	0
15	0.3	2
16	0.2	1
17	0.5	3
18	1.1	10
19	1.3	20
20	0.9	8
21	0.2	1
22	0.2	1
23	0.7	5
24	0.7	5
52	0.2	1
26	0.5	3
27	0.5	3
28	0.8	7
29	0	0
30	0	0
31	0	0
32	0.5	3
33	0.1	1
34	0.3	2
35	0	0
36	0.4	2
37	0	0
38	0	0
39	0	0
40	0	0

*NI = Neutralizing Index.

eight embryos with each dilution, permitting the selection of five living embryos at the time of examination for infectivity. Since the SN range of immune birds is relatively narrow, the endpoint of neutralization can usually be obtained with the use of serum-virus mixtures at three virus dilutions.

In examining the CAM for plaques, care should be taken not to confuse spurious lesions with specific virus plaques. Virus plaques are gray, circular, opaque lesions with smooth edges. Nonviral lesions are frequently seen on the CAM at the point where the punch penetrates the shell membrane and occasionally on other areas of the CAM, possibly due to deposition of shell paritcles. The nonviral lesions are usually yellwish, granular in appearance, and the margins are irregular with radiating projections. After a little experience, one can differentiate the two types of lesions with a high degree of accuracy.

SUMMARY

1. A procedure is described for conducting an SN test with LT virus, employing a dropped-CAM inoculation technique and calculating the EID_{50} endpoints by the method of Reed and Muench.

2. A frozen virus suspension maintained a stable titer when held frozen at -40 C for a period of three months.

3. A comparison of untreated and heat-inactivated serums showed no marked differences in the SN titers obtained.

4. Holding the serum-virus mixtures at room temperature for at least one hour resulted in maximum neutralization.

5. The use of serum diluted 1:2 or 1:5 gave SN titers comparable to undiluted serum after correction for the dilution factor.

6. Pools of serum gave SN titers comparable to mean titer of the individual serum samples.

7. Virus-neutralizing antibodies were detectable in vaccinated birds one week after vaccination and reached their maximum by the third week.

8. The SN test rarely detected ten or more neutralizing doses in serum from LT susceptible birds.

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STUDIES OF THE EFFECT OF DIHYDROSTREPTOMYCIN PROPYLENE-GLYCOL SPRAY ON CHRONIC RESPIRATORY DISEASE IN CHICKENS

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CHRONIC respiratory disease was observed in Israel as early as 1940. The disease was introduced into pedigreed flocks by imported hatching eggs and disseminated by them to most of the chicken farms in the country. Kligler and Perek[13] described the disease, which differed in its symptoms from infectious bronchitis and from laryngotracheitis, and named it erroneously "tracheitis" because of the turbid and

cohesive exudate found in the tracheae of the affected birds. The same authors proved in 1942 the transmissibility of the causative agent by artificially contaminated egg shells. The above mentioned disease has now been identified as CRD infection by its symptoms, lesions and by isolating PPLO in culture. Mixed bacterial invasion has also been observed.

With the reports from Johnson,[11] Van Roekel *et al.*[21] Gross and Johnson,[7] Wong and James,[23] Yamamoto and Adler[24] on the inhibiting effect of different antibiotics upon mortality in CRD-infected chicken embryos, the use of high-level antibiotic feeding of affected flocks was introduced on a large scale in this country. No significant beneficial influence upon mortality in severely affected flocks could be observed. Where high-level antibiotics were fed or administered parenterally at the appearance of the first symptoms of the disease, a stimulating effect on feed consumption, and some reduction in mortality rate might be seen. The reported investigations by Cover,[4] Crawley and Fahey,[3] White-Stevens and Zeibel,[22] Jungherr and Luginbuhl,[72] Olesiuk, Van Roekel, and Beninato,[16] and others reveal very variable results on the influence of antibiotics upon experimental and natural infections.

Hazelhoff,[10] who gave us a clearer insight into the functions and structure of the airsacs, which are sparsely supplied with blood-vessels, indirectly promoted the idea to convey the antibiotics by the spray method into the surrounding air, causing the birds to inhale it into their affected airsacs. Although the spray method† is not new, very little experimentation has been reported.

In order to investigate the effect of the DHS-Propylen-Glycol combination on CRD, the authors carried out two preventive experiments. In addition, two cases of CRD infected flocks treated by the same method are described.

MATERIALS AND METHODS

The material used for the spraying contained per liter of solution: 50 gm of dihydrostreptomycin-sulfate, 300 ml of propylene-glycol, 1 gm of mentholatum and distilled water

† Vet Strep Spray—Merck, Sharpe and Dohme.

ad 1000 ml. Spraying was performed by an electric whirl sprayer in two experiments, and by a simple handsprayer in the other experiments. In each experiment, drug levels and time of exposure varied according to the severity of the disease, and the age of the birds. In the preventive experiments, we sprayed the one-day-old birds in their delivery boxes five times during half an hour, for approximately one minute per spray. A second spray was applied to the chicks at the age of 3½ to 4 weeks at night, when the chicks were concentrated under the hovers.

One liter of the solution was srayed over approximately 2000 day-old chicks, and 2 liters of the solution were used for the same number of birds at the age of 3½ to 4 weeks.

In the two treatment cases, the birds were concentrated in groups in the corners of the chicken houses and each group was sprayed about one minute, repeated five times over half an hour.

EXPERIMENTAL

Experiment 1. This preventive experiment was performed at the Kfar Menahem poultry farm for 60 days. A flock of 3413 day-old New Hampshire, and White Plymouth Rock chicks was divided into two groups, a treated one of 1700, and a control group of 1713. Both groups were housed in two large pens close together. The same attendant worked with both groups. Under the conditions of this farm, it was impossible to house the birds isolated from each other. All the birds were fed a good quality starter mash of 26 percent protein for the first 2 weeks, while in their grower mash, the protein percentage was reduced to 21 percent. A premix of vitamins, coccidiostats and minerals ad optimum requirement, including also 4 gm of procaine-penicillin per ton was mixed into the mash. Deep litter of fresh wood shavings was used on the floor. The hovers were heated by steampipes placed around the walls of the rooms.

The experimental group was sprayed with one liter of the drug solution in their delivery boxes which were grouped in one corner of the house. After treatment, the chicks were

placed under the hovers to dry. The same day, vaccination against fowlpox was performed, as usual. At the age of 3½ weeks, vaccination against Newcastle disease with a live virus vaccine, administered intramuscularly, was performed, and the following evening, the experimental group was treated again with the drug spray. At the age of 43 days, both groups of birds were removed to the broiler house into two separate pens next to each other. During the 60 days of the experiment, the chicks of both groups were carefully observed for respiratory symptoms during the day and also at night. At the age of 35 days, distinctive symptoms of CRD appeared in the control group, and slowly spread within the flock. Airsac lesions were found in some of the autopsied birds. In the treated flock, no CRD symptoms or lesions were observed during the 60 day duration of the experiment. Symptoms of CRD in a light form did appear in the treated group 10 days after conclusion of the experiment. Up to 60 days, 169 birds (9.8 percent) of the control group succumbed, while in the treated group, only 61 chicks (3.5 percent) died during this period.

At the end of the experiment, all the birds were weighed in groups of 15. Data and results are listed in table I. The even growth of the treated flock was surprising, while many runts were seen in the control group. The weight gains analyzed by statistical methods showed a significant difference between the treated and non-treated groups.

TABLE I

Mortality and average weight gains in grams of treated and untreated groups, at 60 days of age

	No. chicks	Death losses	White Rocks m	White Rocks f	New Hampshires m	New Hampshires f
Treated	1700	61 (3,5%)	933	788	981	797
Untreated	1713	169 (9,8%)	912	731	882	781

Experiment 2. This experiment was carried out at the same farm as experiment 1 with 33,129 day-old chicks of the following breeds: New Hampshires, White Rocks, New Hampshire x Leghorn crossbreds. The experimental flock of 17,407

chicks, and 15,722 chicks serving as controls were kept in separate brooder houses, which were similar in construction and size, and within a distance of 50 meters. Each house consisted of ten equal rooms, every one capable of accommodating 1700 birds. In the experimental group, approximately 1740 birds of all three breeds were kept in each pen, while in the control group, 1570 birds of all breeds were kept in each pen. Both houses were serviced by the same road. The management, feed, and water source were essentially the same. A special attendant cared for each house. Prior to the experiments both houses had been cleaned and disinfected thoroughly, and fresh wood shavings were spread on the floor.

The chicks of both groups were vaccinated against fowlpox when one day old. On the same day, the birds of the experimental flock were sprayed with the drug in the same manner and quantity as in the previous experiment. At the age of 4 weeks, the birds of both groups were vaccinated with live Newcastle vaccine, and two days following this vaccination, the experimental flock was treated with a second spray of the drug. At 45 days of age, both flocks were transferred to grower houses where they were kept under strictly identical conditions.

Initial symptoms of CRD infection were first observed among 3½ week old birds in all pens of the control flock. Four days later, during the application of the Newcastle vaccine, light CRD symptoms were seen in a number of birds in one pen of the treated flock; however, 2 days after the second drug-spray application, the clinical symptoms disappeared. No further indications of CRD symptoms could be observed until the birds were 8 weeks old, and then only in a very light form among a relatively small number of birds.

It has to be pointed out, that in all of the affected birds in the control group, from 3½ to 7 weeks of age, the disease did not seem to be of a severe nature, though in several of the older affected birds nasal discharge accompanied the other symptoms and the disease appeared in a more serious form.

At the age of 8 weeks, the birds of both flocks were weighed by random sampling in groups of 15 birds. Ten

groups of males and ten groups of females from each breed were weighed separately. Mortality rates up to 8 weeks and the average weight results are given in table II.

TABLE II

Mortality and average weight gains in grams in birds of treated and untreated flocks at the age of 8 weeks (Experiment 2)

	No. birds	Mortality	W. Rocks m	W. Rocks f	N. Hamp. m	N. Hamp. f	NHxLeghorn m	NHxLeghorn f
Treated	17407	522 (3%)	990	836,5	965,5	822,5	980	735,5
Untreated	15722	803 (5,1%)	841	741	900	732,5	840	662

As seen from table II, the weight gains of the treated birds of all breeds, both males and females, were distinctively higher than the weight gains of the controls. The greatest response to the treatment, as measured by weight gains, was shown by White Rock males which gained 17.7 percent more than their controls, while the response of the New Hampshire x Leghorn males was the lowest among all treated groups, 5.7 percent over that of their controls. The average weight gains of all treated birds were 11.1 percent greater than the controls.

TABLE III

Analysis of weight ranges of experimental and control groups at 8 weeks of age (Experiment 2)

Groups	Mean weight in kg: Groups of 15 birds	Standard deviation	Coefficient of variation	Range in kg
W. Rock m. treated	14.585	0.860	5.9	14.340-14.850
m. controls	12.625	2,631	20.7	11.600-13.600
f. treated	12.572	0.842	6.7	12.280-12.840
f. controls	11.115	1.852	16.6	10.400-12.510
N. Hamp. m. treated	14.362	0.574	4.0	14.180-14.540
m. controls	13.507	3.346	24.7	12.780-15.040
f. treated	12.337	0.293	2.3	12.210-12.420
f. controls	11.062	2.056	18.6	10.100-11.500
NH Leg. m. treated	12.592	0.320	2.5	12.705-12.480
m. controls	12.130	1.040	8.5	11.590-12.670
f. treated	10,447	0.404	3.8	10.200-10.695
f. controls	9.710	0.860	8.8	9.150-10.720

As the control birds exhibited an uneven growth, standard deviations and coeffecients of variation of the weights of both flocks were analyzed. This analysis indicated a much greater degree of scatter in all three control groups, both males and

females, than in the corresponding groups of the treated flock. The data are shown in table III. The difference in weight gains between the treated, and the control groups were highly significant ($P<0.01$).

Because of the appearance of a light CRD infection in the treated flock at the age of 8 weeks the weight gains for the two succeeding weeks are given in table IV.

TABLE IV

Average weight in grams, at the age of 10 weeks of treated and control birds (Experiment 2)

	White Rocks		New Hampshires		NH x Leghorn	
	m	f	m	f	m	f
Treated	1438	1129.5	1445	1168	1235.5	1007.5
Untreated	1234.5	1084	1307.5	1072.5	1145	925

Statistical analysis of the results in table IV indicated the same level of significance as the results obtained at 8 weeks.

Experiment 3. Treatment was performed in a broiler flock kept in batteries and suffering from CRD infection. Eighty severely affected birds at the age of 9 weeks were chosen for this treatment. The birds showed severe symptoms of the disease, such as watery eyes, coughing, sneezing, tracheal rales, listlessness, and ruffled feathers. Several birds had a mucous discharge from their nostrils.

Forty birds of this group were taken for treatment, while the others served as controls. The treament was carried out in a 2½ square meter closed cubicle which was attached to the pen. The birds were sprayed over their heads with the drug five times during half an hour for approximately one minute per spray. After treatment, the birds were removed to a battery adjoining that of the 40 control birds. During the first 24 hours after spraying, four of the treated birds died, while 13 birds of the control group succumbed. The remaining 36 treated birds showed signs of recovery, and two days after

treatment, their appetite improved considerably enough, so that, at 12 weeks of age, they could be marketed at a relatively normal weight. Of the 40 controls, only 20 birds survived. No additional birds of the treated group died up to marketing.

Experiment 4. A flock of 2000 birds kept on deep litter was found to be infected with CRD. In spite of high-level antibiotics being fed, daily mortality amounted to losses of 10 to 20 birds. From this flock, 300 birds which appeared to be severely infected were chosen and treated with the drug spray in a closed room in the same manner as in the previous experiments. Up to the second treatment after 48 hours, 14 birds died. Mortality dropped to zero 24 hours after the second treatment. During the same time the 1700 non-treated birds suffered a mortality of 79 chickens and during the following week 101 died.

DISCUSSION

The reports of Kligler and Perek,[13] Adler *et al.*,[1] Fahey and Crawley[5] furnish the evidence of the transmissibility of the etiological agent of CRD through the contaminated eggshell and transovarially. Due to these established facts, the authors found it proper to start the spray treatment with day-old chicks hatched from infected eggs, as a possible preventive measure.

Grumbles and Boney,[8] Olson,[17] Glover[6] have shown the susceptibility of the PPLO agent to streptomycin. Because other airborne bacteria may be involved, as invaders of the airsacs, we decided to combine the di-hydrostreptomycin spray with propylene-glycol. Robertson and associates[18,19] demonstrated that glycol vapors are rapidly lethal to bacteria in the air. Propylene-glycol was shown to be highly active in vitro as a germicide and of very low toxicity to animals and men. A concentration of 0.5 mg of propylene-glycol vapor per liter of air is, according to Robertson, lethal within seconds to many airborne bacteria. According to Mellody and Bigg,[51] Rosebury *et al.*,[20] the vapors of propylene-glycol are also toxic to fungi and to viruses. A significant reduction of airborne infections by glycol vapors was repeatedly claimed by Harris

and Stokes,[9] Bigg *et al.*,[2] and Lossli *et al.*[14] Mentholatum was added to the spray combination as an indicator for duration of suspension of the vaporized drug.

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SUMMARY

1. In two experiments dihydrostreptomycin-propyleneglycol sprayed on chicks at one-day, and at 3½ to 4 weeks was of value in preventing the clinical symptoms of CRD up to 8 weeks of age. In one experiment slight infection in a treated group occurred after this time.

2. Although the disease was not of a severe nature in the control birds of both experimental flocks, it was shown that the difference in weight gains of the corresponding spray-treated flocks were highly significant ($P<0,01$).

3. Mortality rates of the treated flocks were 3.5 percent against 9.8 percent in the controls of the first experiment, and 3 percent against 5.1 percent in the controls of the second experiment.

4. The growth of the treated flocks was more uniform than that of the untreated ones. Standard deviations and variation coefficients for the weights of both flocks showed a much greater degree of scatter in all the control breeds, both males and females, than in the corresponding groups of the spray treated flocks.

5. In two outbreaks, treatment of heavily affected birds with the described spray methods had a beneficial effect.

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IMMUNIZATION FOR THE CONTROL OF AVIAN ENCEPHALOMYELITIS

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AVIAN encephalomyelitis (AE) is a wide-spread[1] egg-transmitted[3,7] virus[2] disease of chickens that is often costly to hatcherymen because losses from it are largely confined to the first two weeks after hatching, a period during which many hatcheries guarantee livability of their chicks. During a typical outbreak, from 1 to 50 percent of the chicks develop paresis, manifested as a progressive weakness of the legs until the chick is unable to move. A fine tremor of the head and extremities is frequently seen. If not destroyed by the poultryman these chicks usually die from starvation and from beng trampled by their broodmates. Some chicks less severely affected may continue to reach feed and water, survive to maturity and often develop into good layers.

Until recently the disease was believed to have no noticeable effect upon laying hens. However, in an outbreak studied by Taylor, Lowry and Raggi[6] a brief pause in egg production was noted immediately after the laying of a few eggs from which clinically affected chicks were subsequently hatched.

The belief that survivors of an outbreak may act as carriers of the disease has led in the past to the general recommendation that neither the survivors nor the parents of infected chicks should be used for breeding. In an earlier

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paper, Schaaf and Lamoreux[5] presented evidence that the presence of the virus of AE on the premises of a flock of breeding chickens need not interfere with the continued use of that flock for the production of chicks. In the last outbreak of AE that occurred in the winter of 1949-1950, almost all the infected chicks were produced by pullets. Of even greater significance was the fact that the pullets and old hens were housed together in pedigree mating pens where both age groups had equal opportunity for exposure. This suggested that the old hens had been immunized to the disease by a previous exposure. The outbreak subsided in each of the two affected flocks in about three weeks, indicating that immunity developed promptly.

These observations led to the attempt to avoid such trouble in the future by immunizing breeding stock during the rearing period. For this purpose the strain of AE virus isolated from the brains of affected chicks during the outbreak was propagated. Immunization was scheduled for prospective breeding stock at 14 to 18 weeks of age and for old hens at the beginning of their annual forced molt. Over the last six years more than one million doses of live AE virus vaccine have been used in these flocks with no major outbreak of the disease occurring among the chicks produced.

The purpose of this paper is to describe 1) a comparison of the efficiency of different routes of vaccination and 2) the effect of contact exposure upon resistance to subsequent intracerebral challenge.

MATERIALS AND METHODS

Host chicks. The chicks were all progeny of AE-vaccinated pullets and hens. They were held in small buildings separate from the main flocks. Some were on floor litter and some in batteries. They were not vaccinated for any other disease. One man cared for all the chicks, and customarily serviced the houses containing control chicks before caring for the vaccinated groups.

Virus preparation. The AE virus isolated from the 1949-1950 outbreak was used in these tests. It has now undergone

35 brain-to-brain serial passages in chickens 1 to 30 days of age. These were inoculated intracerebrally with 0.05 ml or 0.1 ml of a 10^{-1} brain suspension in nutrient broth containing 5 mg of dihydrostreptomycin per ml. Chicks more than 2 weeks old received the larger dose. Signs of the disease, ataxia progressing rapidly to paresis, appeared in some of the chicks by the 9th to 12th day after inoculation, and by the 21st day most of the intracerebrally inoculated birds had succumbed. Severely affected chicks when unable to reach feed and water were destroyed, their heads were removed and stored at —20 C.

The heads were removed from the freezer, dipped in 99 percent isopropyl alcohol, allowed to dry, and the brains removed aseptically. Various diluents, (nutrient broth, 0.85 percent saline solution, distilled water, 50 percent glycerine and 60 percent glycerine) have all given satisfactory results. An equal volume of diluent was added to the pooled brains and the mixture ground for two minutes in a homogenizer. The mixture was frozen, thawed, and reground in the homogenizer for five additional minutes. This procedure was repeated three times after which the suspension was distributed into rubber stoppered or screw capped vials and stored at —20 C.

Titration of virus. In order to arrive at an approximation of the pathogenicity of the different pools of virus, they were titrated by inoculating young chicks intracerebrally with ten-fold dilutions. The minimum lethal dose for 50 percent of the chicks (CLD_{50}) was calculated according to the method of Reed and Muench[4] as cited by Van Rooyen and Rhodes.[8]

Technique of inoculation. The wing-web vaccination was done with the common, two needled instrument, with a longitudinal groove on each needle. For each vaccination it was dipped into the virus suspension and then thrust once through the web of the wing from the under side. Approximately 0.01 ml of vaccine was delivered with each use of the instrument.

Wing-muscle and thigh muscle vaccinations were performed in the same manner as for the wing-web route, except that the needles were thrust through the flesh between the radius and ulna, and the muscles of the thigh respectively.

For breast-muscle vaccination, intramuscular injection of 0.2 ml of a 1:20 dilution of the virus in broth was done with a syringe fitted with a three-quarter inch 24 gauge needle.

The per os dose was contained in 1.0 ml and was delivered into the esophagus with a two inch blunt, curved, 18 gauge needle attached to a 10 cc. syringe.

Intracerebral challenge. The same virus pool used for vaccination was also used to challenge the immunized birds. Each challenge dose diluted in nutrient broth was contained in 0.2 ml. Five mg of dihydrostreptomycin per ml was added to the inoculum. The challenge inoculations were made with a one inch 20 gauge needle fitted to a 2 ml syringe. The point of the needle was inserted about one-quarter inch at the external canthus of the eye, and was then pushed through the bone of the orbit, directed diagonally inward and backward, coming to rest in the cerebral hemisphere of the opposite side. The thin bone of the orbit offered little resistance and rarely clogged the needle.

EXPERIMENTAL

Experiment 1. For this experiment 157 White Leghorn female chicks were divided in four decks of a battery brooder. When the chicks were 12 days old, the 40 occupying the top deck were vaccinated in the wing web with approximately 138 CLD_{50} and the 40 in the next deck below were vaccinated in the breast muscle with the same quantity of virus per chick diluted in nutrient broth as described above. The 77 chicks in the two lower decks were left as uninoculated controls. Thirty days later, all three groups were challenged intracerebrally with approximately 19 times the quantity of virus used for vaccination. At the time of challenge, all groups were mixed together in one floor pen where they remained until the experiment was terminated. For the prevention of acute coccidiosis, sulfa-quinoxaline at the level of 0.025 percent was administered in the feed. The experiment was terminated 42 days after challenge.

Experiment 2. For this experiment 128 White Leghorn

female chicks were reared together on the floor. When they were 51 days old, 32 were vaccinated in the wing web, 32 in the breast muscle, 32 in the thigh muscle, and 32 were left unvaccinated as contact controls. Each bird received approximately 294 CLD_{50}, of virus. Thirty-three days after vaccination, when the chicks were 84 days old, all were challenged intracerebrally with 10 times the vaccination dose. The experiment was terminated on the 37th day following challenge.

Experiment 3. A single pool of virus was used to prepare all the inoculums for this experiment. The CLD_{50} titer of the virus had been determined previously in 29-day-old chicks. For the purpose of this experiment in which different dose levels of vaccine were used, a dilution of the virus containing 100 CLD_{50} per 0.01 ml was designated a 1x dose. Different quantities of virus are indicated as multiples of this dose.

The chicks for this experiment were selected at random from a brood of 668 White Leghorn females, reared on floor litter. When the chicks were 46 days old, 90 were removed to another house, to serve as isolated controls.

The vaccinated groups were distributed as follows: Pen 1 contained 58 chicks vaccinated in the wing muscle by the stick method with a 1x dose of virus, and 21 unvaccinated contact controls. Pen 2 contained 59 chicks vaccinated in the wing muscle by the stick method with a 2x dose of virus, contained in 0.01 ml, and 18 unvaccinated contact controls. Pen 3 contained 24 chicks vaccinated per os with a 13.3x dose of virus in 1.0 ml, 25 chicks vaccinated per os with a 13,333x dose of virus (the maximum possible dose) in 1.0 ml, and 21 unvaccinated contact controls.

Fifty-seven days after vaccination all groups in this experiment were challenged intracerebrally. The 0.2 ml challenge dose of 17,733 CLD_{50}, was the same for all groups. The chicks vaccinated with a 1x, 2x, 13.3x or 13,333x dose were challenged with 177, 88.6, 1.0 and 0.013 times their vaccination dosage, respectively.

RESULTS

Occasional deaths occurred from coccidiosis and piling,

as well as a few from the traumatic injury incurred in the challenge inoculation. Such mortality was excluded from the final tabulation.

Experiment 1 (see fig. 1)

Following vaccination at 12 days of age, in the wing web, 1 chick among 40 (2.5 percent) developed AE after an incubation period of 19 days, and 1 among 40 (2.5 percent) vaccinated in the breast muscle developed AE after an incubation period of 17 days.

Challenge on the 30th day after vaccination produced mortality from AE in 72 of 77 (93.5 percent) of the isolated control chicks with incubation periods ranging from 10 to 21 days; in 28 of 38 (73.7 percent) wing-web vaccinated chicks with incubation periods ranging from 13 to 22 days; and in 1 of 32 (3.1 percent) breast-muscle vaccinated chicks with an incubation period of 15 days.

Experiment 2 (see fig.2)

Following vaccination at 51 days of age in the wing web, 1 of 32 (3.1 percent) chicks developed AE after an incubation period of 31 days; of those vaccinated in the breast muscle 4 of 25 (16 percent) developed AE after 17 to 25 days; of those vaccinated in the thigh muscle, 3 of 32 (9.4 percent) developed AE after incubation periods of 18 to 21 days. None of the contact control chicks showed any sign of the disease during this time.

Following challenge on the 33rd day after vaccination, 8 of 32 (25 percent) contact control chicks developed AE after 10 to 25 days; and 4 of 25 (16 percent) wing-web vaccinated chicks developed AE after 12 to 30 days. None of the chicks which had survived intramuscular vaccination succumbed to challenge.

Experiment 3 (see figs. 3 and 4).

The results following vaccination at 46 days of age in the wing muscle are shown in fig 3. With the 1x dose of virus, 2 of 58 (3.4 percent) chicks developed AE after incubation periods of 24 and 44 days. Of those vaccinated in the wing muscle with the 2x dose, 8 of 59 (13.6 percent) developed AE after incubation periods of 19 to 41 days. None of those vaccinated per os developed clinical AE as a result of vaccin-

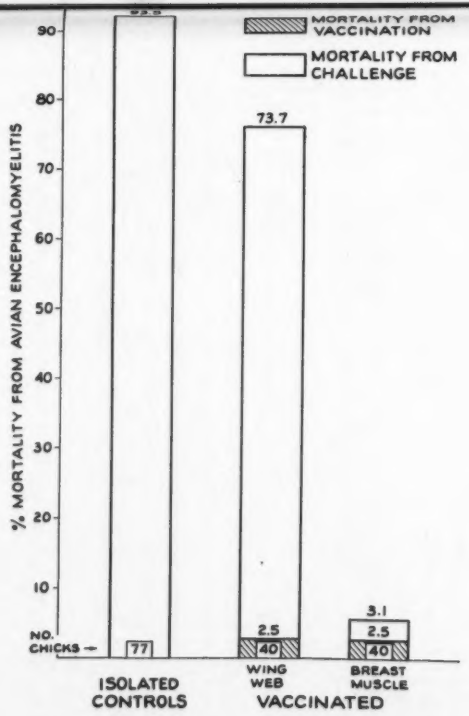

Fig. 1. The incidence of avian encephalomyelitis among control and vaccinated chicks following intracerebral challenge 30 days after vaccination with aproximately 19 times the quantity of virus used in the vaccinating dose. Controls were isolated from the vaccinated chicks until challenged.

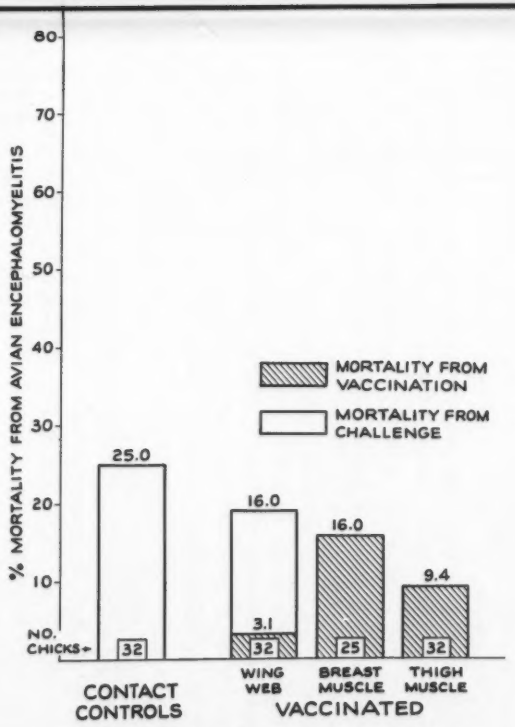

Fig. 2. The incidence of avian encephalomyelitis among control and vaccinated chicks following intracerebral challenge 33 days after vaccination with approximately 10 times the quantity of virus used in the vaccination dose. Control chicks were in contact with the vaccinated chicks throughout the experiment.

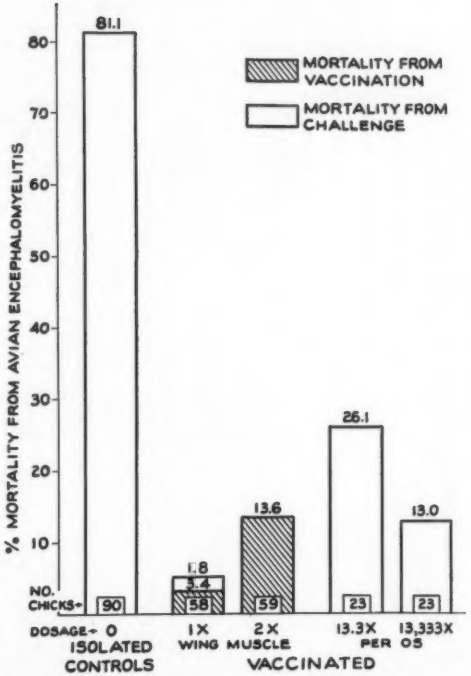

Fig. 3. The incidence of avian encephalomyelitis in experiment 3 among isolated control chicks and chicks vaccinated with different quantities of virus, following intracerebral challenge of the survivors 57 days later.

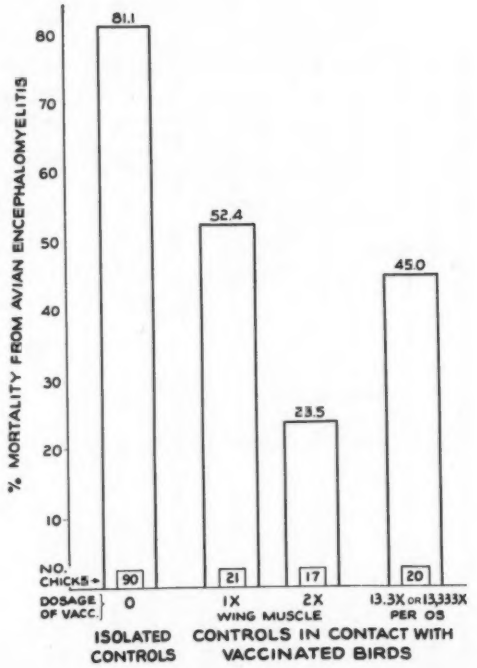

Fig. 4. The incidence of avian encephalomyelitis following intracerebral challenge among isolated control chicks and among control chicks housed in contact wtih vaccinated groups in experiment 3.

ation, and none of the contact controls showed any sign of the disease during this period.

Following challenge administered on the 57th day after vaccination when the chicks were nearly 15 weeks old 73 of 90 (81.1 percent) isolated controls developed AE after incubation periods 11 to 27 days; of the chicks vaccinated in the wing muscle with the 1x dose of virus 1 of 54 (1.8 percent) developed AE after an incubation period of 11 days, and of those vaccinated in the wing muscle with the 2x dose of virus none of 50 chicks surviving vaccination developed AE. Of the chicks which received the 13.3x dose by mouth 6 of 23 (26.1 percent) developed AE after incubation periods of from 11 to 24 days, and of those which received the 13,333x dose 3 of 23 (13.0 percent) developed AE after incubation periods of from 13 to 16 days.

The mortality from AE following challenge inoculation in the different groups of contact control chicks is compared with that in the isolated control group in fig. 4. Among the unvaccinated controls in contact with the chicks which received the 1x vaccination 11 of 21 (52.4 percent) developed AE following challenge after incubation periods ranging from 11 to 15 days, while of those in contact with the chicks vaccinated with the 2x dose only 4 of 17 (23.5 percent) developed AE following challenge, after incubation periods of 13 to 23 days. The 2 groups of chicks vaccinated by mouth, shared the same group of contact controls and among these 9 of 20 (45 percent) developed AE following challenge.

DISCUSSION

It is evident from these results that resistance to AE follows exposure to the virus and that this resistance varies with the method of exposure. Unvaccinated chickens housed with vaccinated ones appear to receive sufficient exposure to develop some degree of immunity without developing signs of AE. Intramuscular vaccination, while producing a stronger and more prompt resistance to intracerebral challenge than contact exposure, or wing-web vaccination, is often followed by more deaths due to vaccination. Resistance following wing-

web vaccination appears to develop slowly. The difference in mortality from AE between isolated controls and wing-web vaccinated chicks challenged 30 days after vaccination is shown in figure 1 to be somewhat less (19.8 percent) than the differences of 46.5 percent and 52.3 percent observed in previous studies when challenged was delayed until 52 and 81 days respectively after wing-web vaccination.[5]

In the tests reported here the mortality from AE resulting from wing-web vaccination (figs. 1 and 2) is considerably higher than that which follows field use of the vaccine. The dosage levels used, and the age of the chicks are factors influencing such mortality. When the wing web dosage is planned for approximately 100 CLD_{50} and vaccination is delayed until pullets are 14 to 18 weeks of age, the losses are generally less than 1.0 percent, based on experience with the virus used in this study.[5]

Vaccination for the control of AE is similar in principle to procedures used with other common virus diseases for which parental immunity in breeding flocks is desirable. AE differs from some others, however, in that vaccination of flocks reared to produce market eggs is apparently neither useful nor desirable. This is because clinical signs of AE are usually lacking or inconspicuous except in young chickens exposed in the egg or perhaps in the incubator, and protection of chicks during the brooding period is afforded by vaccinating the parents.

Whether vaccinated breeeding hens protect their chicks from AE by failing to transmit the virus to the chick, or by passing antibodies through the egg to the chick, or by passing to the chick both virus and neutralizing antibodies is not known. Regardless of the mechanism involved, the protection afforded by wing web vaccination of the dams is the most reasonable explanation for the fact that no major outbreak of AE occurred among the approximately thirty million pullet chicks which have been hatched during the past six years from such breeding stock.

SUMMARY

The results of vaccination of chicks with the live virus of avian encephalomyelitis (AE) by various routes (wing web, intramuscular and per os) and at different dosage levels are described.

Subsequent intracerebral challenge of such vaccinated chicks and their isolated and contact controls showed 1) that the intramuscular vaccination route provided the most protection but also induced the greatest number of clinical cases, and 2) contact controls did not develop visible signs of the disease, and appeared to have acquired some resistance to challenge but not nearly to the same degree as the vaccinated birds.

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THE EFFECT OF ERYTHROMYCIN ON EXPERIMENTAL INFECTIOUS SINUSITIS OF TURKEYS

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PLEUROPNEUMONIA-LIKE organisms (PPLO) can usually be isolated from the sinal exudate of turkeys with infectious sinusitis (IS).[3,4,7] These organisms are generally considered the primary etiological agent in this disease.[2,6] PPLO of avian origin were reported by Yamamoto and Adler[8] to be very sensitive to erythromycin when tested *in ovo*. Hamdy, *et al.*,[5] recently reported the susceptibility of PPLO *in vitro*, *in ovo*, and *in vivo*, to action of various antibiotics, including erythromycin. They stated that erythromycin was the most active antibiotic of those tested *in vitro* and *in ovo*. They were also able to completely eradicate PPLO from the sinal exudate of experimentally infected turkeys with erythromycin medication.

The experiments reported in this paper confirm the reports by Hamdy, *et al.*,[5] and extend some of their observations. The effect of various concentrations of erythromycin and different routes of medication were studied in experimental infectious sinusitis of turkeys. A strain of PPLO, different from that used by Hamdy, was employed.

MATERIALS AND METHODS

A PPLO, strain 91495, isolated from chickens with chronic respiratory disease (CRD), was used for producing IS in turkeys. This strain of PPLO was provided to us through the courtesy of Dr. Jungherr, University of Connecticut.[1] Strain 91495 has been maintained in our laboratory for some time *in vitro* on various culture media. This organism was non-infectious for turkeys until it had been passaged three to four times via the yolk sac in six-day embryonated eggs. The PPLO was then able to kill chick embryos and induce IS in turkeys.

Twenty-four white broad-breasted turkeys, twelve weeks of age, were inoculated intrasinally with PPLO (strain 91495, fifth egg passage). One quarter ml of undiluted egg yolk suspension was injected into each sinus. This dose represented 10^{-7} egg LD_{50} of PPLO per sinus.

Twenty-one days after infection, when the sinuses were swollen, and it was apparent that an infection had been established, the swollen sinuses were aspirated. PPLO was readily isolated from the sinal exudate of each turkey at that time by inoculation of the exudate onto either PPLO agar plates (Difco enriched with sera) or PPLO broth (Difco, enriched with sera). The turkeys were divided into four groups of six and treated as follows:

Group I 50 mg erythromycin intrasinally
Group II 500 mgs erythromycin per ton of feed for 5 consecutive days
Group III 100 mg erythromycin intramuscularly
Group IV Unmedicated controls

The degree of sinus swelling was scored as follows: 0 = no swelling; 1 = slight; 2 = moderate; 3 = large; 4 = very large.

RESULTS AND DISCUSSION

Group I—Intrasinal medication with erythromycin.—The results of intrasinal medication with erythromycin of turkeys infected with PPLO are shown in table I. The untreated turkeys retained approximately the same degree of swelling

TABLE I

Intrasinal medication with 50 mg erythromycin of turkeys infected with PPLO

Turkey #	Treatment	Sinus swelling Before medication		Sinus swelling 12 days post-medication	
		L. sinus	R. sinus	L. sinus	R. sinus
1	None	2	2	2	2
2	"	4	0	3	0
3	"	1	0	1	0
4	"	0	0	0	1
5	"	4	4	4	4
6	"	1	0	1	0
7	Erythromycin Stearate	2	2	0	0
8	" "	4	4	0	0
9	" "	3	3	0	0
10	" "	3	3	0	0
11	" "	4	4	0	
12	" "	2	2	0	0

throughout the experiment (figure 1). The erythromycin treated birds showed no signs of sinusitis twelve days after intrasinal medication (figure 2). The evidence of sinusitis disappeared in the various birds 4 to 12 days after infection. These turkeys then remained free of sinus swelling during the subsequent three-month period.

Group II—Erythromycin in feed.—The second group of turkeys were given erythromycin in their feed at a level of 500 grams per ton for a period of 5 consecutive days. No decrease in the degree of sinal swelling was evident in these turkeys over an observation period of one month. The turkeys in group II were then medicated with erythromycin by intrasinal inoculation. The swollen sinuses of each bird of Group II were drained and then treated with 50 mg per sinus of erythromycin. PPLO was readily isolated from the sinal exudate of these turkeys at that time. It should be noted that these birds had been infected with PPLO for a period of approximately two months prior to intrasinal medication. As seen in table 2, each turkey of Group II was completely free of sinal swelling within ten days following intrasinal medication with erythromycin.

Group III—Intramuscular medication with erythromycin.—The third group of turkeys was treated intramuscularly with erythromycin stearate 100 mg/turkey. These turkeys failed to respond to medication, showing no improvement after 30 days. This group of turkeys was then treated with erythro-

Fig. 1. (left) Turkey with swollen sinuses.
Fig. 2. (right) Turkey after treatment with erythromycin.

TABLE II

Intrasinal medication with erythromycin of turkeys infected with PPLO and medicated previously for five consecutive days with 500 grams erythromycin per ton of feed

	Sinus swelling before intrasinal medication		Sinus swelling after intrasinal medication	
Turkey #	L. sinus	R. sinus	L. sinus	R. sinus
13	0	1	0	0
14	3	3	0	0
15	4	4	0	0
16	3	3	0	0
17	1	1	0	0
18	0	0	Not Treated	

TABLE III

Intrasinal medication with 25, 10, or 5 mg per sinus erythromycin of turkeys infected with PPLO and previously injected intramuscularly with 100 mg erythromycin stearate

		Sinus swelling before medication		Sinus swelling 10 days following intrasinal medication	
Turkey #	Dosage erythromycin per sinus	L. sinus	R. sinus	L. sinus	R. sinus
19	25	0	1	0	0
20	25	4	4	0	0
21	10	3	3	3	3
22	10	4	4	3	3
23	5	1	0	1	0
24	5	3	3	3	3

TABLE IV

Effect of erythromycin injected intrasinally into turkeys 2 months after infection with PPLO

		Sinus swelling before medication		Sinus swelling after intrasinal medication	
Turkey #	Amount erythromycin per sinus	L. sinus	R. sinus	L. sinus	R. sinus
1	25	2	2	0	0
2	25	4	0	0	0
3	25	1	0	0	0
4	25	0	0	0	0
5	25	4	4	2	0
6	25	1	0	0	0

mycin by the intrasinal route of medication. Two birds each were inoculated with either 25 mg, 10 mg, or 5 mg/sinus of erythromycin stearate. As seen in table 3, the swelling of each sinus was completely cleared within ten days after intrasinal

medication with only 25 mg erythromycin per sinus. However, the 10 and 5 mg levels of antibiotic were inadequate to clear the sinuses of infection.

Group IV—Delayed medication with erythromycin.—The degree of swelling in the unmedicated control turkeys did not vary appreciably over the subsequent two-month observation period. This group of animals was then medicated intrasinally with 25 mg/sinus of erythromycin. Four of the five medicated turkeys showed complete recovery after intrasinal medication with 25 mg/sinus of erythromycin (table 4). The sinus swelling was only partially cleared in the fifth turkey.

SUMMARY

Infectious sinusitis was produced in turkeys by intrasinal inoculation of PPLO. Intramuscular medication or addition of erythromycin to the feed was without beneficial effect in this infection. However, the sinal infection could be completely eliminated by intrasinal injection of 50 mg per sinus of erythromycin after the exudate was aspirated.

Intrasinal medication with erythromycin was effective when medication was delayed for at least two months after infections had been established.

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LISTERIOSIS IN FOWLS—A REVIEW

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LISTERIA monocytogenes has been isolated not only from a large number of mammalian species, but also from at least 11 different avian species. These include chicken,[2,4-7,13-16,20,22-25,27-29,31,33,34,36,38,40-42,44-46,48,50,53-57,59-61,64-71] goose,[1,8,29,71] duck,[16,34,71] turkey,[3] pigeon,[10,20] canary,[4,19,63,65] parrot,[20] eagle[49] wood grouse,[32] partridge,[34] and snowy owl.[47] Zink *et al.*[72] and Seeliger[51] also mention the isolation of *L. monocytogenes* from turkeys but without citing published case reports. The above list includes, in addition to the domestic fowl used as a source of food for man, possible house pets, zoo specimens,[10,49] wild game birds and a predator; emphasizing that *L. monocytogenes* is widely distributed in nature. With the increasing recognition of listeria infection both in animals and particularly in man,[51,58] a review of the incidence of this disease in fowls is appropriate; especially since it has been shown that inapparent listeria infection can occur in chickens and that these birds may transmit the infection to man.[13]

A major portion of this paper was prepared while the author was at the Veterinärhygienisches und Tierseuchen-Institut, Justus Liebig-Universität, Glessen, Germany on a Fulbright Research Grant.

Contribution from the Montana Veterinary Research Laboratory (Montana Experiment Station and Livestock Sanitary Board cooperating). Montana State College, Agricultural Experiment Station Paper No. 423 Journal Series.

Characteristics of L. monocytogenes. Listeriosis is caused by *L. monocytogenes*, a rather small diphtheroid, highly pleomorphic Gram positive bacterium. Because of its marked resemblance to some of the so-called "contaminating diphtheroids", hemolytic streptococci, and other members of the Family *Corynebacteriaceae* there is a general feeling that some cultures are discarded before the true identity is established.

The difficulty sometimes encountered in isolating *L. monocytogenes* from certain animal tissues and the ease with which it may be confused with a number of other bacteria, justify the inclusion of a brief description of isolation and identification methods. Although *L. monocytogenes* usually grows well on most of the commonly employed bacterial media, there is strong evidence based on both natural and artificial infections that many times the bacterium fails to grow on initial culture attempts from suspect tissue or body fluids. Csontos *et al.*[8] reported that some cultures prepared from the organs of infected geese failed to show bacterial growth although stained impression smears from the same organs revealed large numbers of bacteria resembling *L. monocytogenes.*

Traub[61] isolated the bacterium from the brains of two mice which had been inoculated intracerebrally with a suspension of liver from a hen apparently suffering from fowl pest. It was felt that the bacterium originated in the chicken liver since *L. monocytogenes* had never been isolated from the mice in this colony on any other occasion. This technique of inoculating laboratory animals with suspensions of tissues or body fluids suspected of harboring *L. monocytogenes* is widely employed in the Soviet Union.[51,58]

A method which has proved highly successful for the isolation of *L. monocytogenes* has been described by Gray *et al.*[17,18] This consists in macerating the tissue to be cultured in a mortar, Waring blendor, or tissue grinder together with sufficient sterile distilled water or nutrient broth to make a suspension. Saline should be avoided since it may have a harmful effect on the bacterium, especially if the population is very small. A portion of this suspension is plated on tryp-

tose agar (Difco) and incubated at 37 C for 18 to 24 hours. When the plates are examined with a binocular scanning microscope and obliquely transmitted illumination as described by Henry,[21] the colonies of *L. monocytogenes* can be readily identified. They are round, slightly raised with a finely textured surface, entire margin, translucent, blue-green in color and watery in consistency.[18] These colonies are distinctive from any other colonies commonly observed in cultures prepared from animal tissues, body fluids and exudates. When initial cultures from suspect material fail to reveal the presence of *L. monocytogenes* the prepared suspensions, or body fluids, should be refrigerated at 4 C and recultured at intervals for a period of at least 2 months. This method, although slow, greatly enhances the probability of isolating the bacterium and has proved successful for the isolation of *L. monocytogenes* from a wide range of animal species in various parts of the world.[58] The cause of this delayed growth is not understood but appears to involve more than mere growth at 4 C[54] which is known to occur.

In general, the various techniques which have proved most successful for the isolation of *L. monocytogenes* stress the need for tissue maceration. This may be due to the frequent intracellular position of the bacterium. Olson *et al.*[39] in a comparison of the efficacy of various isolation methods found that grinding duplicate samples in tryptose broth yielded the best results. Zink *et al.*[72] favored culturing pieces of tissue in thioglycolate broth. More recently Simon[53] recommended a preliminary culture in either brain-heart-dextrose broth, placenta broth or thioglycolate-serum medium.

Several rather consistent and distinctive characteristics of *L. monocytogenes* may be employed in the identification of suspect cultures. Most strains display a rapid tumbling motility when incubated at 18 to 20 C but are rarely motile when grown at 37 C. Many strains produce a marked purulent conjunctivitis following instillation into the conjunctival sac of a rabbit. This is best accomplished by washing off with 5.0 ml of distilled water the growth from a tryptose agar slant culture which has been incubated at 37 C for 18 to 24 hours.

Two or three drops of this suspension are instilled into the conjunctival sac by means of a Pasteur pipette, without scarifying the conjunctiva. When rabbits are inoculated intravenously with a minimal lethal dose of *L. monocytogenes*, after 48 to 72 hours there is usually a marked increase in the monocyte percentage in the circulating blood. If the growth on a tryptose agar slant which has been incubated at 37 C for 18 to 24 hours is suspended in sterile distilled water to a density which corresponds to the number one tube of the McFarland nephelometer, a 0.5 ml inoculation will usually not produce death before the increase in monocyte percentage is evident. Necropsy of these animals often reveals a liver completely studded wtih small, grey-white foci of necrosis. Although these tests may not be employed effectively with all recently isolated cultures of *L. monocytogenes*, they are sufficiently characteristic to be of value in confirming the identification of suspect cultures.

Distribution. As shown in table 1, listeriosis in birds has been reported from all continents except Africa and Antarctica. In general, the disease is reported most commonly from the temperate zone of both hemispheres with extremes ranging from the Arctic[47] to Ceylon.[1] In the United States, the disease has been reported from New York to California and it is almost certain that the true incidence is much higher than the nine published reports indicate. As in other animal species, only the more northern states are represented. This has been a rather consistent finding and may be more than mere coincidence. From the number of published reports it appears that the Netherlands has the highest incidence of listeria infection in birds. However, this is not a reliable measure of the extent of a still somewhat unrecognized infectious disease. Also, it is impossible to arrive at a definite figure of the exact number of birds involved in each report since often only one or a few of a rather large number of birds are submitted for examination. It is rather striking that in Norway where the incidence of listeria infection in mammals is fairly high, *L. monocytogenes* has been isolated only once from a bird, a canary.[19]

TABLE I

Isolation of *L. monocytogenes* from fowls

Species	Author and reference	Year	Country
Chicken			
	Seastone[50]	1935	USA—New Jersey
	Paterson[41,42]	1937/39	England
	Forgeot *et al.*[14]	1941	France
	Cole[7]	1941	USA—New York
	Hurt *et al.*[23]	1941	USA—Illinois
	Pallaske[40]	1941	Germany—Saxony
	Hoffman & Lenarz[22]	1942	USA—California
	Traub[61]	1942	Germany—Hesse
	Lesbouyries[31]	1943	France
	Pothmann[46]	1944	Germany—East Prussia
	Wramby[70]	1944	Sweden
	Muth[38]	1944	USA—Oregon
	Peperkamp and Jansen[44]	1947	Netherlands
	Sparapani[55]	1947	Argentina
	Zeller[71]	1949	Germany—Hesse
	Bigland[4]	1950	Canada—Alberta
	Pletneva & Stiksova[45]	1950	USSR—Moscow
	Bolin & Turn[5]	1951	USA—North Dakota
	Chadkowski & Czarnowski[6]	1951	Poland
	Felsenfeld[13]	1951	USA—Illinois
	Schoop[48]	1951	Germany—Hesse
	van Dorssen & Jansen[67]	1951	Netherlands
	Geurden & Devos[15]	1952	Belgium
	van Ulsen[68]	1952	Netherlands
	Jaarverslag, Drenthe[24]	1952/53	Netherlands
	Jones[27]	1953	Australia—New South Wales
	Juslin and Stenberg[28]	1954	Finland
	Thompson[60]	1954	USA—District Columbia
	van der Vlerk[66]	1954	Netherlands
	van Ulsen[69]	1954	Netherlands
	Lucas *et al.*[33]	1955	France
	Stenberg and Hämmäinen[56]	1955	Finland
	Thal[59]	1955	Sweden
	Jaarverslag, Drenthe[25]	1955/56	Netherlands
	Bastar *et al.*[2]	1956	Czechoslovakia
	Harnach *et al.*[20]	1956	Czechoslovakia
	Kornilova[29]	1956	USSR—Novosibirsk
	Mayer[36]	1956	Germany—Bavaria
	Stricker *et al.*[57]	1956	Czecvhoslovakia
	van der Schaaf *et al.*[64]	1956	Netherlands
	Goyon & Lecomte[16]	1957	France
	Lucas & Seeliger[34]	1957	France
	van der Schaaf *et al.*[65]	1957	Netherlands
Goose			
	Zeller[71]	1949	Germany—Hesse
	Bandaranayake[1]	1953	Ceylon
	Csontos *et al.*[8]	1955	Hungary
	Kornilova[29]	1956	USSR—Novosibirsk

TABLE I (Continued)

Duck		
Zeller[71]	1949	Germany—Hesse
Goyon and Lecomte[16]	1957	France
Lucas and Seeliger[34]	1957	France
Turkey		
Belding & Mayer[3]	1957	USA—Michigan
Pigeon		
Harnach *et al.*[20]	1956	Czechoslovakia
Donker-Voet[10]	1957	Netherlands
Canary		
Bigland[4]	1950	Canada—Alberta
Grini[19]	1951	Norway
van der Schaaf & de Jong[63]	1951	Netherlands
van der Schaaf *et al.*[65]	1957	Netherlands
Parrot		
Harnach *et al.*[20]	1956	Czechoslovakia
Eagle		
Schultz[49]	1950	Germany—Saxony
Wood grouse		
Lilleengen[32]	1942	Sweden
Partridge		
Lucas & Seeliger[34]	1957	France
Snowy owl		
Reed[47]	1955	Canada—Quebec

Symptoms and gross lesions. As in mammalian species, the young fowl appears to be more susceptible to listeria infection than older birds. Outbreaks are usually sporadic and mortality in the individual flock may vary from a few birds to as much as 40 percent of the flock.[8,40,41,48,60] There are no pathognomonic symptoms or lesions in birds with listeriosis. Paterson[41] reported that adult chickens usually died suddenly while in young birds there was a slow wasting before death. In contrast, Csontos *et al.*[8]stated that among geese, the very young (2 to 3 weeks old) died suddenly, often within a few hours after onset of symptoms. Older geese (6 to 8 weeks old) survived somewhat longer and dislpayed torticollis, spasms and other nervous symptoms before death. There may be an increase in the percentage of monocytes in the peripheral blood of naturally infected birds. However, it appears that this is

not a consistent finding and has little diagnostic value. Nevertheless, this is a common reaction in artificially exposed birds, suggesting that this may occur in birds with primary listeria infections, but not in those in which *L. monocytogenes* plays only a secondary role.

Although the isolation of *L. monocytogenes* from fowls is often associated with some other disorder there are numerous reports of what appear to be primary listeria infections.[1-4,7,8,10,14,15,19,20,28,33,38,48,50,57,63,65,66,68,70] In these birds listeriosis is most commonly manifested by a septicemia and the bacterium can be isolated from most of the viscera, particularly the liver or spleen. Occasionally it also has been isolated from the lungs, kidneys and brain. Usually the most conspicuous lesions are massive areas of myocardial degeneration with marked engorgement of the cardiac vessels, pericarditis and an increased amount of pericardial fluid. In some instances, the heart may show varying numbers of well defined greyish-white foci of necrosis ranging in size from pin-point to 5 mm in diameter. At times the heart may show extensive dilatation, particularly of the right side. The apex may be thickened giving the appearance of a "round heart" as is seen in the so-called round heart disease. These cardiac lesions are often erroneously considered not only specific but also characteristic for listerosis of fowls. However, to-day it is well established that similar lesions are found in other species, particularly artificially infected guinea pigs, and that not all birds with listeriosis show this lesion. Focal hepatic necrosis without cardiac alterations also is a common finding in naturally infected birds.[3,4,15,20,33,41,48,63,68] Sometimes these foci are also seen in the spleen and lungs. Other lesions frequently encountered include splenomegaly, nephritis, peritonitis, enteritis, ulcers in the ilium and ceca, generalized or pulmonary edema, inflammation of the air sacs, conjunctivitis. In acute cases, the necrotic lesions tend to be less marked and only congestion and a few small hemorrhages throughout the viscera are seen.

Although septicemia appears to be the most common form of avian listeriosis, there are a few reports in which the predominant symptoms and lesions were related to the

central nervous system. This has been observed in chickens by Muth[38] and van der Vlerk[66], in a goose by Bandaranayake[1], and in a turkey by Belding and Mayer.[3] The most conspicuous symptom in these birds was marked torticollis. In addtion to this, the goose tended to walk in circles and the turkey showed coarse tremors of the skeletal muscles. At necropsy only the goose showed myocardial necrosis. Unfortunately, only Bandaranayake[1] mentions histological findings in the brain. He observed marked congestion, but no foci of infiltration. However, in all these instances *L. monocytogenes* was isolated only from the brain. These reports establish that listerosis in fowls may be manifested not only by a septicemia but also by a localized encephalitis as is commonly observed in ruminants. With our increasing knowledge of listeria infections, it is no longer possible to associate a specific syndrome with a given animal species as was sometimes done in the past. Today, almost all the commonly recognized manifestations of listeria infection have been reported in the various susceptible animal groups.[51,58]

Histopathology. There is a paucity of information on the histopathology of naturally infected fowls. This may be due partially to the fact that often *L. monocytogenes* is isolated rather incidentally from birds which obviously were suffering from some other disorder. Csontos *et al.*[8] found focal necrosis without a marked infammatory reaction in the liver and spleen of geese. The adjacent tissue was either normal or showed only early degenerative changes. The myocardium contained connective tissue fibers and "... a faintly staining homogeneous substance poor in nuclei-surrounded mostly by slight cellular infiltrations...." which consisted of histiocytes, a few polymorphonuclear leucocytes and lymphocytes. In a few instances, there were perivascular cuffing and focal necrosis surrounded by polymorphonuclear leucocytes and lymphocytes in the cerebellum and medulla oblongata. Justin and Stenberg[28] reported hyperemia and fatty degeneration in the myocardium and necrosis in the liver of birds from which *L. monocytogenes* had been isolated. Thomp-

son[60] observed massive fibrosis and collections of heterophiles in sections of chicken liver from which *L. monocytogenes* was isolated. There was also marked proliferation of the bile ducts. The spleen contained almost no lymphocytes and consisted merely of loosely arranged lymphoblasts. There was extensive necrosis bordered by infiltration of lymphocytes throughout the myocardium. The kidneys showed large areas of fibrosis and degenerative changes in the epithelium of the tubules. Many tubules were occluded and distended by casts of degenerated cells. Some of these casts contained Gram positive bacilli. These changes, particularly the massive fibrosis, had not been described previously for listeriosis of fowls and suggests that the disease may be of long duration in chickens. This particular bird had been severely ill about one month before and was the seventh of a group of 12 birds to die. Lilleengen[32] observed fatty degeneration and necrosis of the hepatic cells in liver sections from a wood grouse which at necropsy showed necrosis in the ceca, swollen spleen and greyish discoloration of the liver.

The most detailed description of the histopathology of artificially infected chickens is given by Pallaske.[40] His results and also those of numerous other authors suggest that myocardial degeneration with focal or diffuse necrosis is the most consistently observed alteration following artificial exposure of chickens; providing the birds live for 5 to 7 days. Often there is also a marked pericarditis with an increased amount of pericardial fluid or exudate. Pallaske[40] distinguished between small, medium and large myocardial foci. The first involved only a few fibrils within a muscle bundle. These stained a bit more deeply with eosin and the nuclei showed pyknosis and karyorrhexis. Moderate fatty changes were also evident. The medium foci involved several bundles with complete necrosis at the center and coagulation necrosis at the periphery and some proliferation of hisitocytes. The large foci were characterized by complete destruction of extensive areas of the heart, edema, marked proliferation of histiocytes, infiltration of monocytes, lymphocytes and plasma cells. These often formed a marked "cuff" around the capil-

laries and small vessels. Many of the erythrocytes in these vessels showed degenerative changes in the nucleus. In Gram stained sections, the bacteria were always found at the periphery rather than in the center of the lesion. Seastone[50] found the bacterium within the muscle fibrils and arranged parallel to them as though extension occurred by way ofthe muscle tissue. Pallaske[40] also reported early focal necrosis in the liver and spleen but could not detect lesions in the kidneys or brain. Traub[61] found a swollen spleen with several large white foci but no cardiac lesion in a hen which died 15 days after intravenous exposure and which had shown symptoms of a nervous disturbance. Histologically, a low grade purulent meningitis was observed. Although the intraperitoneally infected turkey poults of Malewitz *et al.*[35] displayed drooping of the head followed by nervous syptoms, hunched position and finally almost complete paralysis and death within 24 to 80 hours after exposure, only a few of the brains showed perivascular cuffing. There was no evidence of inflammation or necrosis. Rod shaped bacteria were seen in circumscribed areas of the brain. The most pronounced lesions were in the livers. These were congested, the sinusoids distended, and there were areas of coagulation necrosis surrounded by infiltration of lymphocytes and macrophages. Large numbers of gram positive rod shaped bacteria were found in the liver, particularly within the Kupffer cells. In the heart there was slight congestion, subpericardial hemorrhage, and congestion of the pericardium with infiltration of mononuclear cells. Bacteria were found in these areas of infiltration. The myocardium appeared normal.

Association with other disorders. It has been suggested often that *L. monocytogenes* plays only a secondary role in fowls. In support of this, listeria infection in birds has been associated with salmonellosis,[33,41] Newcastle disease,[5,33] fowl pest,[61] coryza,[23] coccidiosis,[15,33,64,68] worm infestations,[23,33,61,68] mites,[4] enteritis,[44,68] lymphomatosis,[22,41] ovarian tumor,[33] and lowered resistance due to inbreeding[27] or hatching of imported eggs.[1] The bacterium also has been isolated from the spleen of apparently normal chickens.[13] Reed[47] isolated *L. monocyto-*

genes from the intestional tract of an apparently normal snowy owl shot in the Artic region of Quebec, Canada. This bird may have fed on lemmings which are known to harbor *L. monocytogenes.*[51] The blue eagle from which Schulze[49] isolated *L. monocytogenes* did not appear ill immediately before death. However, it had been treated over a longer period of time for an abscess in the abdominal wall. This was healed about 3 weeks before death. Today it is known that *L. monocytogenes* may be associated with abscesses[58] and this may have been the primary focus of infection. At necropsy the only lesion was a marked swelling of the liver.

Epidemiology. The origin and mode of spread of all listeria infections are still poorly understood. It has been suggested frequently that wild rodents may play an important part in the spread of the disease, but there is presently little substantial evidence to support this postulation.[51,58] Guerden and Devos[15] encountered listerosis in a chicken flock 15 months after an outbreak of meningitis which killed three of eight pigs raised on the same farm. The brains of the dead pigs were not cultured and the remainder were immediately sold for slaughter. Nevertheless, they hinted that this may have been a possible source of infection for the chickens. These authors also felt that contact played only a very small part, if any, in the transmission of the disease in poultry. Csontos *et al.*[8] isolated *L. monocytogenes* from the inflamed conjunctiva of several geese with listeriosis. However, they felt that the presence of the bacterium on the conjunctiva played no part in transmission of the disease. Dedié[9] allowed intravenously exposed birds to run with nonexposed birds. After a period of 2 weeks, antibody titers in the birds exposed by contact ranged from 1:40 to 1:320 and at 6 weeks titers were as high as 1:1280. Nevertheless, they showed no symptoms of illness and the bacterium could not be isolated from any of the birds showing high titers. It appeared that the bacterium was transmitted through the feces and nasal secretion. In support of this opinion Bastar *et al.*[2] observed an increase in antibody titer in chickens exposed orally to *L. monocytogenes,* but did not mention any other reaction as a result of this exposure.

Considerable evidence suggests that *L. monocytogenes* may lead to saprophytic existence and can survive in soil for long periods of time. Among fowls, this concept is supported by the reports of Cole,[7] Felsenfeld,[13] and Bandaranayake.[1] If birds, weakened by physical or physiologic alterations, contact these organisms in the soil, infection may result. That this may be true is suggested by Bandaranayake[1] who observed listeriosis only in geese hatched in Ceylon from eggs imported from England. Local chicks raised under similar conditions in an adjoining brooder were not affected. He postulated that the goslings may have contacted the bacterium in the soil and were infected because of their lower level of resistance. Lesbouyries[31] cites as evidence of the saprophytic existence of *L. monocytogenes* its presence on the conjunctiva of "normal" animals. This claim has been made often, especially among Soviet investigators. However, this has not been confirmed by western investigators.

The fact that listeria infection in fowls is often complicated by some other disorder and the relative difficulty of artificially infecting healthy birds suggest that they possess a rather high degree of natural resistance to listeria infection. Segre *et al.*[52] failed to incite infection in one to two weeks old chicks when antimetabolites were administered in an attempt to alter the tricarboxylic acid cycle. Malewitz *et al.*[35] succeeded in infecting turkey poults only when a relatively large number of organisms was used. This has been the experience of almost all investigators who attempted to artificially infect birds. It appears that among fowls, the canary is the most susceptible to listeria infection and this bird has been successfully employed as a laboratory animal by a number of investigators.[44,51]

Traub,[61] Bolin and Turn,[5] and Geurden and Devos[15] suggested, but without strong support, that latent listeria infection in birds may be more frequent than is generally recognized and that these latent infections may play an important part in the epidemiology of listeriosis in other species. However, in several instances, the owners of infected birds also had a rather large number of other susceptible animals such

as sheep and cows. In no instance was listeriosis reported among them. In the outbreak reported by Geurden and Devos[15] 5 percent of a flock of 80 chickens died of listeriosis, yet in another flock of 1200 chickens only 150 feet away and cared for by the same persons, no deaths due to listeria infection occurred. The relatively high agglutinating titers against *L. monocytogenes* found in many apparently normal birds,[2,9,50,58] tend to support the concept of latent infections in birds. However, the significance of these titers is still controversial.[58] Geurden and Devos[15] showed that sparrows and pigeons could be infected artificially and thus might play a part in transmission of the disease. Pigeons previously had been reported to be refractory to artificial infection with *L. monocytogenes*,[28,31,41] but subsequently the bacterium has been isolated from natural infection in this bird.[10,20] Van der Schaaf and de Jong[63] mentioned that four pigeons kept by the owner of the canary from which they isolated *L. monocytogenes* had died just previous to the death of the canary. Unfortunately these were not submitted for necropsy.

The possible presence of latent carriers in a chicken flock presents a definite public health hazard. Felsenfeld[13] reported an outbreak of listeria conjunctivitis in two poultry processing plant workers. *L. monocytogenes* was isolated from the spleens of five apparently healthy birds which were being processed. It was found that these birds originated from the same area where Hurt *et al.*[23] had diagnosed listeriosis four years before. This is one of the few instances where a definite mode of listeria infection in man could be traced to infection in animals or birds. This report also suggests that *L. monocytogenes* may persist on the same premise for long periods of time. This is further supported by Cole[7] who described eight cases of listerosis in chickens and an additional 18 cases approximately one year later in birds raised on the same ground.

Listeria conjunctivitis in man has been reported on numerous occasions in the Soviet Union.[30,45,51,58]. Many of these reports stress patient contact with sick birds or birds which recently died of undetermined causes. Also in central

Europe many women giving birth to infants infected with *L. monocytogenes* had recent contact with sick or dead birds. [30,51,58] Although there is considerable incriminating circumstantial evidence that birds may transmit listeria infection to man only the report of Felsenfeld[13] has been confirmed.

Possible transmission through eggs. Geurden and Devos[15] suggest that eggs may be a possible source of listeria infection. They isolated *L. monocytogenes* from extensive necrotic lesions in the oviduct of one hen. Unfortunately, no eggs from this bird were available for culture. Nevertheless, three children under five years of age eating prepared eggs from this flock remained healthy and a 35 year old man who ate raw eggs from this flock showed no signs of illness. Forgeot *et al.*[14] also isolated the bacterium from the contents of an "ovule", necrotic myocaridum and bone marrow of a chicken which had died. Schoop[48] also mentioned extensive lesions in the oviduct of hens from which *L. monocytogenes* was isolated.

Although unconfirmed, transmission of listeria infection through eggs presents interesting epidemiological possibilities. Hartwigk[58], Kampelmacher[58], and Urbach and Schabinski[62] cultured numerous eggs from various sources, but failed to isolate *L. monocytogenes.* Dedié[9] cultured 26 eggs from nine chickens owned by a woman who, 4 weeks previously, had given birth to an infant infected with *L. monocytogenes.* During the next 4 weeks, the period in which the eggs were collected, the H agglutinating titer of the chickens increased from 1:40 to 1:60 up to 1:80 to 1:320 and the O titer increased from 1:20 to 1:80 up to 1:40 to 1:160. Although *L. monocytogenes* was not isolated from any of the eggs, the author felt that the chickens may have been the source of infection for the woman. Dedié[9] also could not isolate *L. monocytogenes* from any of 200 eggs laid by hens during a 10 week period following either intravenous or contact exposure to the bacterium. However, during this period the bacterium was isolated from the feces and nasal secretion of infected birds. This finding suggests that although the contents of the eggs laid by hens infected with *L. monocytogenes* may not necessarily contain the bacterium, eggs contaminated

by fecal material may constitute a source of infection if not properly cleaned before use. Urbach and Schabinski[62] in a series of investigations with artificially infected eggs found that the number of viable *L. monocytogenes* cells increased approximately 500,000 times in eggs incubated at room temperature for 6 to 10 days. Neither the appearance nor the odor of these eggs were altered. Guinea pigs fed with these infected eggs after a period of starvation developed a fatal listeria septicemia. When these eggs were fried so that the white was congealed but the yolk was still soft, *L. monocytogenes* could be isolated from them. There was also an increase in the number of viable *L. monocytogenes* cells in eggs stored under refrigeration. Thus the egg may constitute a possible source of listeria infection.

Artificial infection of embryonating eggs. There are several reports of the artificial exposure of embryonating chicken eggs to *L. monocytogenes*. Paterson[43] found numerous, discrete, white, pin-point foci on the chorio-allantoic membrane 36 hours after intra-allantoic inoculation of 14-day-old embryonating chicken eggs. Lesions developed only in the endoderm and mesoderm. The ectoderm was not altered. After 48 hours' incubation, small, white foci of necrosis could be observed in the liver and heart, and after 60 hours there was evidence of meningitis. Jaeger and Myers[26] produced death in 24 to 48 hours following intra-allantoic inoculation of 10-day-old chicken embryos. At the site of inoculation, there were pox-like lesions on the chorio-allantoic membrane. Dontenwill and Knothe[11] reported that the large, white foci on the chorio-allantoic membrane and the focal, hepatic necrosis which develop after 48 hours incubation in 10-day-old embryonating chicken eggs inoculated on the chorio-allantoic membrane with material suspected of harboring *L. monocytogenes*, are so characteristic that this can be employed as a rapid diagnostic aid. They also reported thickening of the ectoderm in addition to lesions in the endo- and mesoderm, but could not detect lesions in the brain. Mencikova[37] found that 18-day-old embryonating chicken eggs inoculated lightly with *L. monocytogenes* on the chorio-allantoic membrane

hatched normally. Although the bacterium could be isolated from the chicks and their feathers, most of them survived and developed normally. However, when large inocula were employed, the chick hatched normally, but developed encephalitis and died within the first 5 days of life.

Dedié[9] found that by the inoculation of embryonating chicken eggs with rough cultures of *L. monocytogenes*, reversion to the smooth form took place.

Eveleth *et al.*[12] succeeded in isolating *L. monocytogenes* from the cerebro-spinal fluid of sheep by inoculation of suspected material into 10-day-old chick embryos. Dedié[9] and Segre *et al.*[52] also suggest this technique for attempts to isolate *L. monocytogenes*. With the established difficulty sometimes encountered in attempts to isolate *L. monocytogenes*, this may prove to be a useful isolation method and deserves further investigation.

Prevention. The present widespread use of poultry feeds containing antiotics may be an effective prophalytic measure against listeria infection. This was suggested by Csontos *et al.*[8] During a large outbreak of listeriosis among young geese, a group of 360 birds were given feed containing "Aurofac". This was administered at the rate of 5 to 20 mg of aureomycin per kg of body weight. Of this group, only 1.1 percent of the birds died during the two week observation period. In the untreated control group of 100 birds 24.0 percent died during the same period.

SUMMARY

The incidence of *Listeria monocytogenes* infection in fowls, the symptoms displayed, and the lesions observed are reviewed. The numerous publications reveal that there are no pathognomonic symptoms or lesions for the infection since the bacterium is often associated with some other disorder in the bird. Primary listeria infection is characterized most often by a septicemia with myocardial necrosis and/or focal hepatic necrosis. Occasionally a localized encephalitis with or without gross lesions is encountered. There is strong circumstantial evidence that birds may play an important role

in the transmission of the bacterium to other animals and man. The embryonating chicken eggs may be of value in attempting to isolate *L. monocytogenes* from suspected material. Feed containing antibiotics may be an effective prophylactic against listeria infection in birds.

The most commonly employed methods for isolating and identifying cultures of *L. monocytogenes* are also reviewed.

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EXPERIMENTAL PSITTACOSIS AND ORNITHOSIS IN TURKEYS: COMPARISON OF NINE STRAINS

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LESIONS in turkeys, from which an agent was isolated and later identified as ornithosis virus, were first reported by Boney *et al.*[2] Later, Pate *et al.*[7] described the lesions in naturally and experimentally infected turkeys. Beaudette *et al.*[1] and Dickinson *et al.*[5] each reported the lesions observed in turkeys naturally infected with ornithosis. Page[6] described the gross lesions in turkeys experimentally infected with an ornithosis agent (New Jersey strain). Davis and Delaplane[4] described the gross lesions produced by the Jo strain ornithosis virus in turkeys from one through 24 weeks of age.

The purpose of this paper is to report the symptoms, mortality, and gross lesions produced in turkeys by each of nine strains of psittacosis or ornithosis virus.

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MATERIALS AND METHODS

The strains of ornithosis and psittacosis viruses used in this study, the sources from which they were isolated, the agencies making the isolation and identification, and the date of the original isolation were as follows:

6BC strain	parakeet	Hooper Foundation	1941
56-69 strain	parakeet	Texas Agr. Exp. Sta.	1956
56-68 strain	parrot	Texas Agr. Exp. Sta.	1956
57-5 strain	turkey	University of Minnesota	1956
57-6 strain	turkey	University of Minnesota	1956
56-57 strain	turkey	Oregon State College	1956
56-237 strain	turkey	Texas Agr. Exp. Sta.	1956
56-253 strain	turkey	Texas Agr. Exp. Sta.	1956
Ku strain	turkey	Texas Agr. Exp. Sta.	1954

All of the viruses used in this study were propagated in chicken embryos and were fifth serial passages except for the 6BC strain and the 57-6 strain. The number of passages to which the 6BC strain had been subjected is unknown. The 57-6 strain was in the eleventh embryo passage. Each turkey was inoculated by the intratracheal route with 0.25 ml of the amnio-allantoic fluid containing the desired strain. Simultaneously, the viability of each inoculum was tested by passage in chicken embryos.

About 300 day-old broad breasted bronze turkey poults were used in this experiment. When the poults were 3 weeks of age, 90 were removed for the first phase of the experiment; at 10 weeks of age, 90 poults were removed for the second phase; and at 18 weeks of age, 54 turkeys were removed for the third phase.

The procedure for all phases of this experiment was similar. The turkeys were divided into nine equal groups. Each group was isolated in a separate room in a security-type laboratory and inoculated with one of the strains of virus. After a 14-day observation period, all survivors were killed. All turkeys were necropsied and the lesions recorded. At the end of each phase, 5 uninoculated turkeys were removed from the supply flock and utilized as controls.

Attempts to reisolate virus from the spleens of turkeys

inoculated with 6BC, 56-59, 56-68, 57-5, and 57-6 strains were conducted in chicken embryos. The tissues were considered negative if virus could not be recovered by two passages. In addition, attempts were made in mice to reisolate the virus from spleens of turkeys inoculated with the 6BC strain.

Representative tissues from all turkeys were fixed in Zenker's solution and stored in alcohol for histopathlogical studies.

RESULTS

The results of this study are summarized in table 1. The results from an earlier experiment with the Jo strain ornithosis virus[4] are included in table 1 for comparison; however, in this earlier study the observation period for the 3 and 10-week-old turkeys was only 7 days.

Reduced feed consumption, slight depression, and some yellowish-tinted droppings were observed in 3-week-old poults inoculated with strains 6BC, 56-69, 56-68, 57-5, and 57-6. Clinical evidence of disease was not seen in older birds inoculated with these strains. Anorexia, respiratory distress, bright yellow droppings, and depression were observed in all groups inoculated with strains 56-57, 56-237, 56-253, and Ku; however, more severe symptoms were observed in 3-week-old poults than in older birds.

There was no mortality in the 3-week-old poults inoculated with strains 6BC, 56-69, 56-68, 57-5, and 57-6; however, the mortality rate was 80 percent or more in 3-week-old poults inoculated with strains 56-57, 56-236, 56-253, and Ku. One 10-week-old poult died in the group inoculated with strain 56-57 and two in the 56-237 group. No other mortality occurred.

The gross lesions produced by all strains differed only in severity. The 56-57, 56-237, 56-253, and Ku strains produced more severe lesions than did the other strains. Predominant lesions were caseo-fibrinous pericarditis, caseo-fibrinous peritonitis and aero-sacculitis, perihepatitis (plastic exudate), biliary stasis, and splenomegaly. The incidence of these lesions for each strain and age group are given in table 1. Table 1

TABLE I

Summary of results of inoculating turkeys of three ages with each of nine strains of psittacosis or ornithosis virus

Strain	Symptoms	% Mortality	% Pericarditis	% Peritonitis and aerosacculitis	% Perihepatitis (Plastic exudate)	% Biliary Stasis	Average Spleen Size in mm
			Three-week-old turkeys				
Uninoculated	0	0	0	0	0	0	12.4
6BC	Slight	0	40	60	0	0	13.8
56-69	Slight	0	40	83	0	0	15.7
56-68	Slight	0	90	60	0	10	16.0
57-5	Slight	0	80	90	20	10	15.0
56-6	Slight	0	70	60	0	0	16.0
56-57	Severe	80	100	60	20	0	17.5
56-237	Severe	90	100	90	0	30	18.6
56-253	Severe	100	90	70	0	0	16.3
Ku	Severe	80	100	100	10	20	16.0
Jo*	Severe	68	85	55	8	0	—
			Ten-week-old turkeys				
Uninoculated	None	0	0	0	0	0	22.0
6BC	None	0	40	50	0	0	21.5
56-59	None	0	20	100	0	0	21.8
56-68	None	0	30	100	0	0	20.7
57-5	None	0	40	100	0	0	21.2
57-6	None	0	10	60	0	0	20.2
56-57	Moderate	10	100	100	0	0	22.7
56-237	Moderate	20	90	100	40	60	26.1
56-253	Moderate	0	60	50	10	50	26.6
Ku	Moderate	0	60	50	10	40	24.1
Jo*	Severe	5	65	58	15	55	28.3

TABLE I (Continued)

		Eighteen-week-old turkeys					
Uninoculated	None	0	0	0	0	0	31.6
6BC	None	0	0	50	0	0	34.7
56-69	None	0	17	67	0	0	36.0
57-5	None	0	33	33	0	0	37.0
56-68	None	0	67	33	0	0	33.7
57-6	None	0	33	67	0	33	34.0
56-57	Moderate	0	67	50	0	0	31.5
56-237	Moderate	0	100	67	17	33	37.5
56-253	Moderate	0	50	17	33	33	44.9
Ku	Moderate	0	100	100	33	87	39.3
Jo*	Moderate	20	90	90	70	50	31.0

*The results of previous experiments with Jo strain ornithosis virus[4] are included for comparison; however, the observation period for 3 and 10-week-old turkeys was one week.

also includes the average size of spleens of the control and infected birds for each group. Splenomegaly occurred more frequently in birds inoculated with strains 56-57, 56-237, 56-253, and Ku. Enlarged spleens often had a whitish, mottled appearance. Pneumonia was usually present in birds that died. The lungs were congested, edematous, and some contained areas of caseous exudate.

The 6BC strain could not be recovered from spleens of turkeys inoculated with that strain. The 56-88 was recovered from 3-week-old poults. The 56-69 and 57-5 strains were recovered from 3 and 10-week-old poults and the 57-6 strain from turkeys of all ages.

DISCUSSION

Based on the effects produced in turkeys, the viruses used in this study may be classified as mildly pathogenic strains (6BC, 56-69, 56-68, 57-5, and 57-6) and highly pathogenic strains (56-57, 56-237, 56-253, and Ku). Two turkey strains (57-5 and 57-6) produced mild reactions in experimental turkeys similar to those produced by viruses of psittacine origin. Highly pathogenic strains produced symptoms, mortality and lesions similar to those produced by Jo strain.[4] Marked clinical evidence of disease was observed in turkeys infected with virulent strains; however, the mild strains produced little clinical evidence of disease in turkeys. The virulent strains used in this study have also been responsible for serious outbreaks of ornithosis in processing plant workers. It seems that the psittacosis-ornithosis agent should be classified according to pathogenicity rather than by the host from which they were isolated.

Recovery of virus from tissues of survivors inoculated with the virulent strains was not attempted because previous experience had shown that these viruses persist in tissue for 14 days.[3] Virus recovery rate was low from birds inoculated with the mild strains indicating that the body defense mechanisms readily overcome these agents. Older turkeys were more resistant to infections than young poults.

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SUMMARY

The effects produced by each of nine strains of psittacosis and ornithosis viruses in turkeys of various ages are described. The character of the gross lesions were the same for all strains; however, four highly pathogenic ornithosis strains produced more severe lesions than the five mildly pathogenic strains. Older turkeys were more resistant to infection than young poults.

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STUDIES ON PASTEURELLOSIS
II. COMPARATIVE EFFICIENCY OF KILLED VACCINES AGAINST FOWL CHOLERA IN CHICKENS

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ATTEMPTS have been made to prevent outbreaks of fowl cholera in chickens ever since the classical experiments of Pasteur,[15] in which he protected chickens against fowl cholera by vaccination. His methods, however, could not be sucessfully duplicated. Since that time, the search for a dependable method of control of this disease has been continued by the employment of prophylactic, therapeutic, and eradicative procedures. None of these methods of control have been satisfactory, and the quest for a better solution of the problem continues.

In the past few years, the writers conducted a series of experiments on vaccination against fowl cholera in which a number of vaccines were prepared. It is the purpose of this report to show that a high degree of immunity can be stimulated and maintained in chickens against fowl cholera with killed *Pasteurella multocida* vaccines.

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REVIEW OF LITERATURE

Hadley[8] was successful in immunizing rabbits against *P. multocida* by subcutaneous inoculation of an avirulent culture followed by inoculation of a virulent culture. This procedure was not satisfactory, however, when applied to chickens.

Mack and Records[13] found that the use of bacterins in infected flocks produced sufficient resistance to check outbreaks of fowl cholera promptly in 15 out of 16 lots of fowls in 14 flocks. One or two injections satisfactorily controlled the outbreak. There was no apparent difference in the results whether homologous or heterologous strains of *P. multocida* were used in the preparation of bacterins.

Van Es and Martin[19] tested six commercial vaccines or bacterins against fowl cholera and concluded that no reliance could be placed on them for the prevention of fowl cholera. However, they still expressed confidence in the possibilities of artificial immunity for the control of fowl cholera.

Hilbert and Tax[10] made an autogenous bacterin from organisms isolated from ducks affected with fowl cholera. The organisms in this bacterin were killed by the addition of 0.5 percent phenol. After 2 years' experience with duck cholera vaccination, they concluded that the product was a very effective agent for controlling cholera in ducks. They found that it was also effective in controlling fowl cholera in a flock of chickens; only 3.7 percent of vaccinated birds died, whereas 32.7 percent of unvaccinated birds were lost.

Carter[3] compared the immunity produced in mice by broth bacterins and chicken-embryo vaccine made from type 1 *P. multocida*. The superiority of the chicken-embryo vaccine as compared with the broth bacterins in the immunization of mice was clearly demonstrated.

Bolin, Turn, and Eveleth[2] inoculated flasks of broth with *Pasteurella* organisms from various sources and incubated them at 37 C until the organisms were dead. The various cultures were then mixed and preserved with formalin. This mixed bacterin was said to be highly immunogenic. The highest degree of immunity was produced in about a week, but it was of short duration.

Dougherty[5] made a comparison of the immunogenicity of seven *P. multocida* bacterins when administered to ducklings. Four were commercial bacterins, and three were made in his laboratory, one of which was a chicken-embryo vaccine. The chicken-embryo vaccine was superior to all other bacterins as it protected the largest percentage of ducklings against challenge with virulent cultures of *P. multocida*.

The use of light mineral oil in water-in-oil emulsified vaccines for enhancing and prolonging antibody production has been comprehensively reviewed by Freund.[6] Bain and Jones[1] described methods for the production of an emulsified hemorrhagic septicemia (*P. multocida*) vaccine. Iyer *et al.*[11] reported that hemorrhagic septicemia vaccine prepared with the addition of mineral oil and lanolin protected a high percentage of calves when challenged with a virulent strain of *P. multocida*.

In preparing an efficient vaccine against *P. multocida*, Priestly[16] emphasized the importance of using a highly virulent and markedly capsulated strain of the organism, and of killing the organisms in such a way as not to alter the capsular antigen. Yaw and Kakavas[21] reported that chickens and mice could be protected with crude preparations of capsular materials from type 1 strain of *P. multocida*.

MATERIALS AND METHODS

New Hampshire chickens from a closed flock were used in all experiments. These birds were free from all known infectious diseases except for a few birds with fowl leucosis and for those birds in experiment 4 which came down with fowl pox in the fall of 1955, before they were vaccinated against fowl cholera. No antibiotics or vaccines were used in raising the breeder flocks or their progeny.

A light mineral oil known as Bayol F was used in the emulsified vaccines and was obtained from Esso Standard Oil Company, Linden 1, New Jersey. The emulsifying agents polyoxyethylene sorbitan monooleate (Tween 81) and mannide monooleate (Arlacel A) were obtained from the Atlas Powder Company, Wilmington, Delaware. Light mineral oil and Ar-

lacel A have been used by several investigators.[7,14,17]

Preparation of Vaccines. A highly virulent strain of *P. multocida* (stock no. X-73), type 1 by Little and Lyon's serological classification[12], was used in all vaccines. This strain was isolated from a peracute case of fowl cholera in a large breeder flock and held in a lyophilized state until needed.

The base suspensions of bacteria for the aqueous and emulsified vaccines were prepared by reconstituting stock culture X-73 from the lyophilized state in 1 percent peptone broth. The suspension was streaked on beef-infusion agar containing 5.0 percent chicken serum and was incubated for 24 hours at 37 C. The agar plates were held for 24 to 48 hours before streaking in order to dry up the condensate within the plates. A smooth, iridescent colony,[4] referred to as "fluorescent" in the first paper[9] of this series, was selected and used to inoculate peptone broth; this seed culture was incubated 6 hours at 37 C, and 2 ml used to inoculate each Roux bottle, which contained 150 ml of dextrose starch agar (Difco.). An additional 1.5 percent agar was added to keep the medium from collapsing after inversion of the Roux bottles. After 24 hours' incubation in an inverted position, the excess seed culture was poured from each bottle and discarded. The bacteria were washed from the agar with 8 to 10 ml of physiological saline and pooled in 500 ml flasks. The base densities were adjusted with a McFarland nephelometer, so that each dose of vaccine contained approximately 3.25×10^{10} bacteria.

Formalin or liquid ethylene oxide was used to kill the bacteria. When ethylene oxide was used, the bacterial suspension was cooled to 2 C before adding the ethylene oxide. The suspension was stored in a cold room overnight at 7 C and then held at room temperature for 24 hours to permit evaporation of any residual ethylene oxide. The ethylene oxide-killed vaccines were preserved with 0.25 perecnt formalin, except those in experiment 1, which were stored in a dry-ice chest until needed.

The emulsified vaccines were processed by mixing oil, emulsifier, and killed bacterial suspension in a Waring Blendor for 4 minutes.

The bacterial suspensions used in the aqueous vaccines of experiments 1 and 2 were diluted to 25 x McFarland 1 density with physiological saline and killed with 1 percent ethylene oxide. These vaccines were labeled "ethylene oxide-killed aqueous vaccine 1 (EAV-1)" and ethylene oxide-killed aqueous vaccine 2 (EAV-2)," respectively. The commercially prepared vaccine used in experiment 2 was purchased in the open market. It consisted of a chemically killed whole broth culture of 50 percent *P. multocida* and 50 percent *Salmonella gallinarum*.

In experiment 3, Carter's method,[3] with some modifications, was used to make the chicken-embryo vaccine (CEV). The chorioallantoic sacs of 8-day-old chicken embryos were inoculated with 0.1 ml of a 6 hour peptone broth culture of *P.multocida*, strain X-73. All embryos died within 12 hours, but were allowed to incubate for 24 hours. The contents of four embryonated eggs were added to 100 ml of physiological saline plus enough formalin to give a final concentration of 0.25 percent. This material was mixed in a Waring Blendor and labeled "chicken-embryo vaccine 3 (CEV-3)." The vaccine was held at 7 C for 1 week after which time a sterility test was made by inoculating 10 ml of thioglycollate medium with 1 ml of vaccine. Iyer's method,[11] with slight modifications was used to make the alum precipitated broth vaccine. Two flasks, each containing 4,000 ml of peptone broth, were inoculated with 5 ml of a 6 hour peptone broth culture of *P. multocida*, strain X-73. After 23 hours' incubation at 37 C, formalin was added to give a concentration of 0.5 percent. The flasks were held 24 hours at room temperature, after which time enough 10 percent potassium alum was added to give a final concentration of 1 percent. The flasks were held at 7 C for another 24 hours, and then all but 1,000 ml of the precipitated culture was passed through a Sharples centrifuge. The precipitate was resuspended in the remaining 1,000 ml of the precipitated culture, and the suspension was labeled "alum-precipitated vaccine 3 (APV-3)."

Comp

Group	Type	Vaccine: Doses: Number	Vaccine: Doses: ml	Vaccine: Doses: Interval in weeks	1 S/C	%
1	EAV-1	1	1	—	9/10	90
2	EAV-1	2	1	2	9/10	90
3*	—	—	—	—	0/5	0
1	EAV-2	2	1	7	—	—
2	Commercial	2	1	2	—	—
3*	—	—	—	—	—	—
1	CEV-3	1	1	—	—	—
2	APV-3	1	1	—	—	—
3*	—	—	—	—	—	—
1	EEV-4	2	1	8	—	—
2	EAV-4	2	1	8	—	—
3*	—	—	—	—	—	—
1	EEV-5	1	0.5	—	—	—
2	FEV-5	1	0.5	—	—	—
3	EAV-5	1	0.5	—	—	—
4*	—	—	—	—	—	—
1	CAEV-6	1	0.5	—	—	—
2*	—	—	—	—	—	—

EEV = ethylene oxide-killed emulsified vaccine.
EAV = ethylene oxide-killed aqueous vaccine.
FEV = formalin-killed emulsified vaccine.
CAEV = capsular antigen emulsified vaccine.
S = number survived.

C
%
*
CEV
APV

TABLE I

Comparison of immunity produced by different vaccines and dosages in experiments 1, 2, 3, 4, 5,

							Weeks postvaccination challenge						
%	2 S/C	%	4 S/C	%	8 S/C	%	9 S/C	%	10 S/C	%	13 S/C	%	S
					Experiment 1								
90	10/10	100	9/10	90	6/10	60	—	—	—	—	—	—	
90	10/10	100	9/10	90	7/10	70	—	—	—	—	—	—	
0	1/5	20	1/5	20	2/5	40	—	—	—	—	—	—	
					Experiment 2								
—	—	—	10/11	91	—	—	—	—	—	—	7/10	70	
—	—	—	0/9	0	—	—	—	—	—	—	1/10	10	
—	—	—	0/10	0	—	—	—	—	—	—	1/11	9	
					Experiment 3								
—	—	—	—	—	—	—	—	—	6/24	25	—	—	
—	—	—	—	—	—	—	—	—	13/32	41	—	—	
—	—	—	—	—	—	—	—	—	0/20	0	—	—	
								Experiment 4					
—	—	—	—	—	—	—	19/19	100	—	—	—	—	
—	—	—	—	—	—	—	19/20	95	—	—	—	—	
—	—	—	—	—	—	—	3/20	15	—	—	—	—	
								Experiment 5					
—	—	—	—	—	—	—	—	—	—	—	29/30	97	
—	—	—	—	—	—	—	—	—	—	—	28/30	93	
—	—	—	—	—	—	—	—	—	—	—	21/29	72	
—	—	—	—	—	—	—	—	—	—	—	0/15	0	
								Experiment 6					
—	—	—	—	—	—	—	—	—	—	—	—	—	3
—	—	—	—	—	—	—	—	—	—	—	—	—	

C = number challenged.
% = percent survived.
* = unvaccinated controls.
CEV = chicken-embryo vaccine.
APV = alum-precipitated vaccine.

TABLE I

ison of immunity produced by different vaccines and dosages in experiments 1, 2, 3, 4, 5, a

Weeks postvaccination challenge														
2		4		8		9		10		13				
S/C	%	S/C	%	S/C	%	S/C	%	S/C	%	S/C	%	S/C	%	S/
Experiment 1														
/10	100	9/10	90	6/10	60	—	—	—	—	—	—	—	—	—
/10	100	9/10	90	7/10	70	—	—	—	—	—	—	—	—	—
/5	20	1/5	20	2/5	40	—	—	—	—	—	—	—	—	—
Experiment 2														
—	—	10/11	91	—	—	—	—	—	—	7/10	70	—	—	—
—	—	0/9	0	—	—	—	—	—	—	1/10	10	—	—	—
—	—	0/10	0	—	—	—	—	—	—	1/11	9	—	—	—
Experiment 3														
—	—	—	—	—	—	—	—	6/24	25	—	—	—	—	—
—	—	—	—	—	—	—	—	13/32	41	—	—	—	—	—
—	—	—	—	—	—	—	—	0/20	0	—	—	—	—	—
Experiment 4														
—	—	—	—	—	—	19/19	100	—	—	—	—	—	—	—
—	—	—	—	—	—	19/20	95	—	—	—	—	—	—	—
—	—	—	—	—	—	3/20	15	—	—	—	—	—	—	—
Experiment 5														
—	—	—	—	—	—	—	—	—	—	29/30	97	—	—	—
—	—	—	—	—	—	—	—	—	—	28/30	93	—	—	—
—	—	—	—	—	—	—	—	—	—	21/29	72	—	—	—
—	—	—	—	—	—	—	—	—	—	0/15	0	—	—	—
Experiment 6														
—	—	—	—	—	—	—	—	—	—	—	—	—	—	30/
—	—	—	—	—	—	—	—	—	—	—	—	—	—	3/

number challenged.
percent survived.
unvaccinated controls.
chicken-embryo vaccine.
alum-precipitated vaccine.

4, 5, and 6.

S/C [15]	%	S/C [16]	%	S/C [22]	%	S/C [25]	%	S/C [39]	%
—	—	4/10	40	—	—	3/13	23	—	—
—	—	8/10	80	—	—	7/10	70	—	—
—	—	0/5	0	—	—	0/5	0	—	—
—	—	—	—	—	—	7/10	70	—	—
—	—	—	—	—	—	0/12	0	—	—
—	—	—	—	—	—	0/10	0	—	—
—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—
—	—	—	—	25/28	89	—	—	—	—
—	—	—	—	36/39	92	—	—	—	—
—	—	—	—	1/20	5	—	—	—	—
—	—	—	—	—	—	24/30	80	30/33	91
—	—	—	—	—	—	25/30	83	20/31	65
—	—	—	—	—	—	3/10	30	7/16	44
—	—	—	—	—	—	0/20	0	0/20	0
30/32	94	—	—	—	—	34/36	94	—	—
3/20	15	—	—	—	—	1/10	10	—	—

In experiment 4, the bacterial suspension was adjusted to 50 x McFarland 1 density and killed with 1 percent ethylene oxide. One hundred and fifty ml of the bacterial suspension were added to an equal volume of saline containing 0.5 percent formalin as a preservative, and the vaccine was labeled "ethylene oxide-killed aqueous vaccine 4 (EAV-4)." Another 150 ml of the killed suspension was added to 141 ml of Bayol F plus 9 ml of Tween 81 and 0.75 ml of formalin. This mixture was emulsified and labeled "ethylene oxide-killed emulsified vaccine 4 (EEV-4)."

In experiment 5, the bacterial suspension was adjusted to 100 x McFarland 1 density. Three hundred ml of this suspension was killed with 1 percent ethylene oxide, and 150 ml with 0.5 percent formalin. To 150 ml of each suspension were added 145 ml Bayol F and 4.5 ml of Arlacel A. These mixtures were emulsified and labeled "ethylene oxide-killed emulsified vaccine 5 (EEV-5)" and "formalin-killed emulsified vaccine 5 (FEV-5)," respectively. The other 150 ml of ethylene oxide-killed suspension was mixed with an equal volume of formalinized saline, and the vaccine was labeled "ethylene oxide-killed aqueous vaccine 5 (EAV-5)."

In experiment 6, the bacteria were harvested and the density adjusted to 75 x McFarland 1 density. To 150 ml of the bacterial suspension were added 150 turbidity-reducing units of hyaluronidase (Wydase) to dissolve the bacterial capsule. The suspension was held at room temperature overnight and then centrifuged at 2,000 r.p.m. for 10 minutes. The supernatant was removed and sterilized by passing through a Seitz filter. An equal volume of Bayol F with 3 percent Arlacel A was added to the filtrate along with enough formalin to give a final concentration of 0.25 percent. This mixture was emulsified and labeled "capsular antigen emulsified vaccine 6 (CAEV-6)."

Vaccination and Challenge. Highly virulent strains of *P. multocida* were used to challenge the immunity. The cultures, which had been isolated from acute cases of fowl cholera, were held in a lyophilized state until needed for challenge, and then one of the strains was reconstituted in peptone broth and streaked on beef infusion agar plates containing 0.5 per-

cent chicken serum. After 24 hours of incubation, a smooth, iridescent colony was selected and replated. If there was no dissociation of colonies after the second plating, a suspension was made of the colonies in peptone broth to a density of 79 percent light transmission on a Bausch and Lomb colorimeter. This was approximately 1.66 x 10^9 bacteria per ml, which was the base concentration for all dilutions of challenge. The exposure dose for experiments 1, 2, and 3 and for the 8 weeks postvaccination challenge of experiment 4 was administered by diluting the base suspension to 10^{-6} with peptone broth and injecting 0.1 ml intramuscularly into each chicken. Consequently, each bird received approximately 166 viable organisms as a challenge dose. All subsequent challenges were made by diluting the base suspension 1:5 with peptone broth and swabbing the nasopharynx of each bird with a cotton swab saturated with the bacterial suspension. Thus, each bird received approximately 0.11 ml of culture as determined by weighing 141 cotton swabs before and after saturation with the challenge culture and reweighing after swabbing the nasopharynx. This was approximately 3.65 x 10^7 bacteria per challenge dose.

All groups of birds, with two exceptions, were confined to finishing batteries for exposure and for 3 weeks thereafter, during which time they were observed for signs of the disease. The two exceptions were the group exposed at 22 weeks in experiment 4 and the group exposed at 39 weeks in experiment 5. The group in experiment 4 was observed for a period of 33 weeks after exposure, and the group in experiment 5 for 9 weeks. All birds that died were necropsied and cultured and all morbid birds were killed and also necropsied and cultured to confirm the diagnosis of fowl cholera as recorded in table 1.

All intramuscular injections of vaccines were made in the leg, and all subcutaneous injections were made in the anterior dorsal area of the neck.

Experiment 1. One hundred and fifty birds, 15 to 25 weeks old, were divided into three groups. Group 1, consisting of 60 birds, was given a 1.0 ml intramuscular injection of

the aqueous vaccine. The second group of 60 birds received two 1.0 ml intramuscular injections 2 weeks apart. The third group of 30 unvaccinated controls were held in isolation until needed for exposure.

The immunity produced from the above vaccinations was challenged with a 0.1 ml intramuscular injection of a 10^{-6} dilution of *P. multocida*, strain X-73. The exposure culture was administered at the following intervals: 1, 2, 4, 8, 16, and 25 weeks. At each of these intervals, 10 birds from each of the two vaccinated groups and 5 from the unvaccinated group were exposed.

Experiment 2. In this experiment there were two vaccinated groups and an unvaccinated control group of 31 birds each. Group 1 was given two doses of the experimental aqueous vaccine intramuscularly. The first dose of 1.0 ml was administered at 12 weeks of age, and the second dose of 1.0 ml was given 7 weeks later. Group 2 received two doses of the commercial vaccine intramuscularly as recommended by the manufacturer—0.5 ml for the first dose and 1.0 ml for the second dose 2 weeks later. The immunity of each vaccinated group was challenged at 4, 13, and 25 weeks postvaccination with *P. multocida*, strain P-705.

Experiment 3. Seventy-six 14-week-old chickens were divided into three groups. Group 1 consisted of 24 birds vaccinated with the CEV, 12 of which received 1.0 ml intramuscularly and 12 received 1.0 ml subcutaneously. In group 2 the APV-3 was administered in like manner, 17 chickens receiving 1.0 ml intramuscularly and 15 receiving 1.0 ml subcutaneously. The remaining 20 were used as unvaccinated controls. The immunity produced by the two vaccines was challenged at 10 weeks postvaccination with *P. multocida*, strain P-912.

Experiment 4. One hundred and forty-six 12-week-old birds were divided into three groups. Group 1 (47 birds) received two 1.0 ml intramuscular injections of the EEV-4, 8 weeks apart; group 2 (59 birds) received two 1.0 ml intramuscular injections of EAV-4, 8 weeks apart; and group 3 (40 birds) was the unvaccinated control. Birds were selected

from each group at 9 and 22 weeks postvaccination, and the immunity challenged with strain P-936 of *P. multocida.*

Experiment 5. Two hundred and ninety-four 12-week-old birds were divided into four groups. Group 1 (93 birds) received 0.5 ml of EEV-5; group 2 (91 birds) received 0.5 ml of FEV-5; group 3 (55 birds) received 0.5 ml of EAV-5; and group 4 (55 birds) was the unvaccinated control. The vaccines were injected subcutaneously. A variable number of birds, as indicated in table 1, were removed from each group at 13, 25, and 39 weeks postvaccination and exposed by swabbing the nasal cleft with *P. multocida,* strain P-936.

Experiment 6. Ninety-eight 12-week-old birds were divided into two groups. Group 1 (68 birds) received one 0.5 ml subcutaneous injection of the CAEV-6. Group 2 (30 birds) was the unvaccinated control. Birds were selected at 15 and 25 weeks postvaccination, and the immunity challenged by swabbing the nasal cleft with *P. multocida,* strain P-936.

RESULTS

Experiment 1 (table 1). It is evident from an inspection of table 1 that two doses of aqueous vaccine were more effective than one in producing a sustained immunity. For the first month there was no difference between a one or two dose vaccination, but from there on to the end of the experiment, the one dose vaccination rapidly lost effectiveness, whereas the two-dose vaccination gave satisfactory protection for the duration of the test.

Experiment 2 (table 1). Group 1 of this experiment, like group 2 of experiment 1, gave satisfactory immunity to the end of the experiment, but it was also a comparative check on the protection afforded by a commercial vaccine. The group of birds vaccinated with the commercial vaccine, however, had no more protection against virulent *Pasteurella* than the unvaccinated group.

Experiment 3 (table 1). This experiment was a comparative test of two vaccines—chicken-embryo and alum-precipitated. Under the conditions of the experiment both vaccines were unsatisfactory.

Experiment 4 (table 1). This was a comparative test of an emulsified vaccine and an aqueous vaccine administered in two doses 8 weeks apart. The emulsified vaccine produced extensive intramuscular, granulomatous inflammation in the injected legs. This appeared to be the result of Tween 81, since Bayol F injected alone did not produce a similar inflammation. However, when Arlacel A was used in place of Tween 81 in experiments 5 and 6 and the vaccine was injected subcutaeously in the neck, no adverse effects were noticed.

Both the aqueous and emulsified vaccines were nearly equal in effectiveness in providing immunity against virulent *P. multocida* organisms up to at least 22 weeks postvaccination. This again demonstrates the superiority of two doses of aqueous vaccine over one.

Experiment 5 (table 1). In this experiment there was no significant difference between the immunity produced by the ethylene oxide-killed vaccine used in group 1 and that produced by the formalin-killed vaccine used in group 2, except at the 39 week challenge. A higher percentage of the birds was protected in group 1 than in group 2. The emulsified vaccines (EEV and FEV) however, were clearly superior to the aqueous vaccine (EAV) in producing a sustained immunity.

Experiment 6 (table 1). This experiment showed that an emulsified cell-free capsular extract of *P. multocida* gave effective immunity against virulent *P. multocida* for at least 25 weeks.

DISCUSSION

The first three experiments (table 1) were conducted to compare the relative merits of one or two doses of vaccine in establishing a lasting immunity, and also to compare the aqueous vaccines prepared by the writers with a commercial vaccine and with vaccines described by other investigators.

In group 2 of experiment 1, the second dose was arbitrarily given two weeks after the first. In group 1 of experiment 2, the vaccinations were administered at 12 weeks of age and

again at approximately 20 weeks to coincide with the usual time of handling the birds, i.e., the first vaccination was administered when the birds were put on the range and the second vaccination, when the pullets were housed. The second vaccination was considered a booster dose given at a critical time. Furthermore, the single vaccination in experiment 1 indicated that adequate protection could be expected for 8 weeks.

In experiment 4, the aqueous vaccine was about as effective in protecting chickens against a strong challenge of virulent *P. multocida* as the emulsified, at both the 9 week and 22 week challenges when given in two doses. However, when only one dose was given, as in experiment 5, it is clearly shown that the emulsified vaccines were superior to the aqueous vaccine.

The reason for the difference between the percentages of survivors vaccinated with the ethylene oxide vaccine and those vaccinated with the formalin-killed vaccine at the 39 week challenge (experiment 5) has not been determined. However, there is a possibility that this discrepancy may have been due to the amount of formalin used to kill the *Pastuerella*. The EEV contained only 0.25 percent formalin as a preservative, but the FEV was killed with 0.5 percent formalin, which was not diluted to 0.25 percent until the oil was added at the time of emulsification. Work is in progress to determine the optimum concentration of formalin required for killing and preserving the *Pasteurella*.

In view of the high percentage of survivors to the severe nasopharyngeal exposure of birds vaccinated nearly 10 months before, it appears that the immunity conferred by a single dose of emulsified vaccine is a substantial one, and that the vaccine would give adequate protection under industrial conditions throughout the laying year. This, however, requires proof by vaccination with control groups under field conditions where natural outbreaks are prevalent or are anticipated.

It is the conviction of the writers that only type 1^{12} strains of *P. multocida* isolated from acute cases of fowl cholera are

necessary in the production of fowl cholera vaccines. Yaw *et al.*[20] also reported that only type 1 is virulent for chickens.

Salk and Laurent[18] reported that influenza vaccine when emulsified could be diluted 100,000 times and still be effective in antibody formation, whereas a hundred-fold dilution not emulsified was ineffective. In view of the immune response stimulated with the aqueous vaccine in experiments 1, 2, 4, and 5, work is now in progress to determine the minimum practical number of bacteria needed per dose of emulsified vaccine to produce and maintain a high level of immunity.

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SUMMARY

Results of studies are reported with six different killed *Pasteurella multocida* vaccines against fowl cholera; one vaccine was commercial and five were experimental (emulsified, aqeuous-suspended, alum-precipitated, chicken-embryo, and capsular).

A single dose of water-in-oil emulsified *P. multocida* vaccine produced an effective immunity against fowl cholera for at least 9 months.

Two doses of aqueous-suspended vaccine were more effective than one in maintaining immunity up to 6 months.

A single dose of emulsified vaccine was superior to two doses of aqueous vaccine in producing an immunity of long duration.

The emulsified capsular vaccine produced an effective immunity for at least 25 weeks.

The immune response stimulated by the alum-precipitated vaccine, the chicken-embryo vaccine, or the commercial vaccine was of questionable value for protection of chickens against fowl cholera, in the dosages and concentrations employed.

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THE ROLE OF PLEUROPNEUMONIA-LIKE ORGANISMS IN INFECTIOUS SINUSITIS OF TURKEYS

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INFECTIOUS sinusitis of turkeys is an important disease problem in all of the major turkey producing areas of the United States. When the lesions are confined to the sinuses, the disease is usually referred to as the "upper form" of infectious sinusitis. If the trachea, lungs, and air sacs are involved it is usually designated as the "lower form" of the disease. In some birds, one form of the infection may predominate while in others both forms may be equally evident. In an affected flock, both forms of the disease are usually present even though one type may predominate.

This disease has been shown to be transmitted through the egg. Before effective control measures for the disease can be formulated or applied, the primary etiologic agent or agents must be established. Some reports indicate that pleuropneumonia-like organisms (PPLO) are the causative agent while others suggest that a viral agent may be the primary

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etiological factor and that pleuropneumonia-like organisms play a secondary role in the occurrence of the disease.

Sinusitis in turkeys was probably first described in England by Dodd[4] in 1905 and in the United States by Tyzzer[20] in 1929. This condition has been described under several names, but the term "infectious sinusitis", suggested by Dickinson and Hinshaw,[3] is almost universally used. This nomenclature excludes miscellaneous conditions and infections that may result in a sinusitis in individual birds. As used in this report, it includes both the "upper" and "lower" form of the disease.

Many investigators have shown that the agent which causes infectious sinusitis of turkeys is identical to the agent which causes chronic respiratory disease (CRD) in chickens. Therefore, it is necessary to consider both diseases in order to obtain a clear understanding of the present state of knowledge concerning the etiology of infectious sinusitis. In 1935 Nelson[18] reported that coccobacilliform bodies were associated with a form of coryza in chickens. These bodies could not be cultivated in artificial media, but could be demonstrated in stained smears made from the exudate from affected birds. In 1943 Delaplane and Stewart[2] reported a virus-like agent as the cause of a chronic respiratory disease of chickens. This disease was described as a slowly spreading infection that persisted in birds for weeks or months. It was characterized by nasal exudation, respiratory rales and a loss in body weight. An agent was isolated from the affected birds that could be propagated in chicken embryos, but not in artificial media. The embryo propagated agent reproduced the typical disease when inoculated into mature chickens.

Johnson[13] reported a respiratory disease that occurred chiefly among broilers 6 to 10 weeks of age. He observed a slight nasal discharge, respiratory rales, and a persistent hacking cough. The lesions were reported to be confined chiefly to the air sacs and lungs. A marked thickening of the air sac membranes and massive accumulation of caseous exudate in the air sac cavities were described. This disease was considered to be closely related to, if not identical, with infectious sinusitis in turkeys.

Minard and Jungherr[17] reported a virus-like agent as the probable cause of an air sac infection in turkeys. These workers considered that they were working with a new condition. The description of this agent and the histopathology[15] induced by it, suggest that this agent was not a PPLO. A virus-like agent as the cause of infectious sinusitis of turkey was reported by Jerstad and Hamilton[12] and confirmed by Hitchner,[10] Hoyt, Pomeroy and Roepke,[11] Prier, [19], and Grumbles and Boney.[28] Groupe and Winn[6] and Groupe, Winn, and Jungherr[7] demonstrated an agent from turkey sinusitis which morphologically resembled the chlamydozoaceae (lymphogranuloma-psittacosis) in producing elementary bodies. This agent was not pathogenic in mice and was reported to be antigenically unrelated to the lymphogranuloma venereum group. Because of additional information that has accumulated since this report was published, the significance of the serological tests performed is in doubt. The evidence indicates that these authors probably were not working with a pure PPLO infection. Delaplane[1] did much to unify the present concept of the etiology of these two conditions when he demonstrated that the egg propagated chronic respiratory disease agent from chickens was capable of causing sinusitis in turkeys.

Markham and Wong[16] and later Van Roekel,[21] Grumbles, Phillips, Boney and Delaplane,[9] and others demonstrated that the etiologic agent of chronic respiratory disease of chickens and infectious sinusitis of turkeys could be cultivated in cell free media and probably belonged to the pleuropneumonia-like group of organisms.

In 1954, Fahey and Crawley[5] reported the isolation of a viral agent from chickens affected with a chronic respiratory diseases. These workers suggested that this virus was probably the primary cause of this disease and that PPLO probably play a secondary role in its etiology. Johnson[14] working with the "Crawley agent", reported that this virus would produce symptoms and lesions in chickens similar to those described for chronic respiratory disease and that air sac tissue suspensions from these chickens, when injected into the sinus of turkeys, produced a sinusitis based on microscopic lesions. The

histopathology in the inoculated turkeys was reported to be identical to that previously described for infectious sinusitis. Pomeroy (personal communication) has isolated ornithosis virus from the sinus exudate of turkeys with sinusitis.

These reports and the inability of many workers to reproduce the chronic respiratory disease-air-sac syndrome in chickens or sinusitis in turkeys with PPLO grown in pure culture in cell free media, have caused confusion as to the true role of PPLO in the etiology of these two diseases. The primary purpose of this study was to clarify the role of the PPLO as the primary cause of infectious sinusitis in turkeys.

MATERIALS AND METHODS

The PPLO used in this study were isolated from cases of chronic respiratory disease in chickens and infectious sinusitis in turkeys that were presented to the poultry disease diagnostic laboratory. Primary isolations were made in phenol-red dextrose broth (Difco) enriched with 2 percent of PPLO serum fraction (Difco). Thallium acetate at a concentration of 1:2000 and 1000 units of penicillin per ml were added to this medium for primary isolation. No inhibitors were used for subsequent transfers. The cultures were incubated at 37.5 C. After the organisms had been isolated, they were maintained and transferred serially in the PPLO serum fraction enriched dextrose broth without inhibitors. Transfers were made with a tuberculin syringe and usually 0.2 ml of culture were added to approximately 10 ml of medium in a cotton stoppered test tube. The cultures were transferred as soon as possible after the dextrose medium was fermented. The time required to ferment dextrose varied from one to 7 days, or sometimes longer with recently isolated cultures.

The PPLO isolates were identified by their characteristic growth and fermentation reaction in carbohydrate media enriched with serum fraction and their inability to grow in carbohydrate media not enriched with the serum fraction or on other standard bacteriological media. Isolates were further identified by their ability to hemagglutinate chicken red blood cells, and their agglutination by known PPLO antisera.

Since these organisms would survive for only a short

period in the culture media used, some inoculations were made into the yolk sac of 7-day-old embryonated chicken eggs. Yolk material was harvested 5 to 7 days later and stored at a temperature of approximately —20 C. Organisms stored in this manner remained viable for several weeks or months.

Each culture of PPLO studied was inoculated into the infraorbital sinus and trachea of two or more turkeys. The inoculated birds were observed for at least thirty days or until symptoms or lesions of infectious sinusitis developed. The inoculated birds in which no symptoms or lesions of infectious sinusitis developed were necropsied at the end of the observation period, and examined for lesions.

When symptoms of sinusitis developed in an inoculated group, sinus and/or tracheal exudate and air sac and lung tissues were collected. One portion of the exudate or tissue suspension was used to attempt to re-isolate PPLO. Another portion was treated with 25 mg of dihydrostretomycin per ml to eliminate the PPLO and then inoculated into embryonated chicken eggs in an effort to demonstrate or eliminate the presence of a viral agent. Since serological tests and histopathological lesions are of unknown reliability, the presence or absence of infectious sinusitis in the inoculated turkeys was based on recognizable symptoms and gross lesions. A number of turkeys of each flock from which birds were used were maintained as uninoculated controls. The turkey inoculations and the manipulations to which each strain was subjected are shown in table 1.

A strain of "Crawley virus" was obtained through the courtesy of Dr. Harr of Dr. Salsbury's Laboratories, Charles City, Iowa. The virus was propagated in the allantoic cavity of 10-day-old chicken embryos. The allantoic fluid harvested from third embryo passage was used immediately to inoculate ten turkeys. Each bird received 0.5 ml of the undiluted fluid inoculated into the infraorbital sinus. Viability of the virus in the inoculum was checked by inoculating chicken embryos.

Because the occurrence or distribution of the "Crawley virus" was unknown, it was considered possible that this agent

might be an accidental contaminant in cultures of PPLO. Studies were designed to determine how long this agent would persist in PPLO media. Each of three tubes containing 10 ml of the serum fraction enriched dextrose media was inoculated with 0.2 ml of allantoic fluid containing "Crawley virus". Serial transfers were made in the same manner as described for the PPLO cultures. Each transfer resulted in a 50 fold dilution of the virus. After 3 days of incubation at 37.5 C, each serial transfer was inoculated into the allantoic cavity of embryonated chicken eggs to check for viability of the agent.

RESULTS

Twelve strains of PPLO were isolated, identified, and their ability to cause infectious sinusitis in turkeys determined. Five of the cultures were isolated from cases of turkey sinusitis and seven were isolated from chickens with symptoms and lesions of chronic respiratory disease or "air sac syndrome". The results of pathogenicity tests are summarized in table 1.

The Ti, T2, T3, T388 and T388a strains were isolated from turkeys. Each of these cultures except 388 and 388a produced typical sinusitis when inoculated into turkeys. The T1 strain was checked for pathogenicity in the 4th, 8th, 12th, 16th, 20th, 43rd, and 100th cultural transfer. Each culture used, except the 100th, produced sinusitis in all birds inoculated. The 100th cultural transfer caused no symptoms or gross lesions of sinusitis.

The T2 strain was isolated from the tracheal exudate of a turkey with symptoms and lesions of the lower form of infectious sinusitis by culturing it directly in PPLO media. The 2nd cultural transfer was inoculated into the sinus of three turkeys and all developed typical sinusitis. The sinus exudate was then aspirated from the inoculated birds and PPLO were isolated again. The organisms were carried through three additional transfers. Again three turkeys were inoculated. One bird developed typical sinusitis.

The T3 strain was isolated from the sinus exudate of turkeys that had been previously inoculated with a suspension of air sac and tracheal exudate from turkeys with symp-

TABLE I

The results of inoculating turkeys with cultures of pleuropneumonia-like organisms

Strain	Inoculum used*	Number turkeys inoculated	Results
T1	Suspension of sinus & tracheal exudate from a field case of sinusitis in turkeys	2	Sinusitis in both turkeys
T1	E 3 C 4-Broth Culture	3	Sinusitis in all 3 birds
T1	E 3 C 8-Broth Culture	5	Sinusitis in all 5 birds
T1	E 3 C 12-Broth Culture	5	Sinusitis in all 5 birds
T1	E 3 C 16-Broth Culture	5	Sinusitis in all 5 birds
T1	E 3 C 20-Broth Culture	5	Sinusitis in all 5 birds
T1	E 3 C 43-Broth Culture	3	Sinusitis in all 3 birds
T1	E 3 C 100-Broth Culture	3	No evidence of sinusitis
T2	C 2-Broth Culture	3	Sinusitis in all 3 birds
T2	C 2 T 1 C 3-Broth Culture	3	Sinusitis in 1 bird
T3	Suspension of air sacs and tracheal exudate from field case in turkeys	2	Sinusitis in both birds

TABLE I (Continued)

T3	C 11-Broth Culture	2	No evidence of sinusitis
T3	C 11 E 3-Yolk material pooled from 3 embryos	2	Evidence of slight sinusitis in one bird at autopsy
T3	C 11 E 4-Yolk material pooled from 2 embryos	3	Evidence of sinusitis in one bird at autopsy
T388	E 1 C 4-Broth Culture	3	No evidence of sinusitis
T388a	E 1 C 1-Broth Culture	3	No evidence of sinusitis
C1	C 3-Broth Culture	3	Sinustitis in all 3 birds
C1	C 3 T 1-Sinus exudate	2	Sinusitis in both birds
C1	C 3 T 2-Sinus exudate	3	Sinusitis in all 3 birds
C2	C 6 E 3 C 3-Broth Culture	3	Sinusitis in all 3 birds
C3	C 4-Broth Culture	3	No evidence of sinusitis
C4	C 4-Broth Culture	3	No evidence of sinusitis
C5	C 5-Broth Culture	3	No evidence of sinusitis
C6	Suspension of tracheal and air sac exudates from typical air sac disease in chickens	3	Sinusitis in all 3 birds
C7	C 7-Broth Culture	3	No evidence of sinusitis

C—Cultural Transfers E—Passage in embryonating chicken eggs T—Passage through turkeys

toms and lesions of the lower form of infectious sinusitis. Two turkeys were inoculated with the 11th cultural transfer of this strain. No symptoms or lesions of sinusitis developed. Embryonated chicken eggs were inoculated with the 11th cultural passage and the organisms were carried through three serial transfers in the yolk sac of chicken embryos. The yolk material from the 3rd embryo passage was inoculated into two turkeys. Mild lesions of sinusitis developed in one bird. Yolk material from the fourth embryo passage was also used to inoculate three additional turkeys. One bird developed lesions of sinusitis.

Strain 388 and 388a were both isolated from the same sample of sinus exudate collected from a field case. Strain 388 was isolated from the fresh sinus exudate while 388a was isolated from the same material after it had been frozen for 46 days. Strain 388 was unique in that it always caused 100 percent mortality within 24 to 48 hours after inoculation into the yolk sac of 7-day-old chicken embryos. Strain 388a caused a mortality that occurred throughout the incubation period. Usually this strain produces a 20 to 40 percent mortality. Although isolated from a typical case of sinusitis, neither strain caused a recognizable sinusitis in the inoculated birds.

The C1, C2, C3, C4, C5, C6, and C7 strain were isolated from chickens with symptoms or lesions of chronic respiratory disease or "air sac syndrome". The third cultural transfer of the C1 strain was used to inoculate three turkeys. All three developed typical sinusitis. The sinus exudate was collected, a portion was cultured for PPLO, and the remaining undiluted exudate was inoculated into two turkeys. Both birds developed sinusitis. The sinus exudate was collected from these birds, cultured for PPLO and a portion of the undiluted exudate was inoculated into three additional turkeys. All three birds developed typical sinusitis. PPLO were isolated from the sinus exudate collected from each of the three groups. The direct transfer from turkey to turkey was done to determine whether pathogenicity would be increased by passage through turkeys. With each successive bird to bird transfer, the incubation period became shorter and the symp-

toms and lesions became more severe indicating that virulence was increased.

The C2 strain was carried through six cultural transfers, three passages in embryos, three additional cultural transfers, and was then inoculated into three turkeys. All three birds developed typical sinusitis.

The pathogenicity of strain C6 was established only on the exudate from the naturally affected chickens from which it was isolated.

The C3, C4, C5, and C7 strains, when inoculated into turkeys, caused no symptoms or gross lesions. The turkeys were destroyed approximately 30 days after inoculation and an attempt was made to isolate PPLO from the trachea, lungs, and air sacs. All attempts were negative.

In each group of experimental turkeys that developed sinusitis, the sinus exudate was collected, cultured for PPLO and inoculated into embryonating chicken eggs in an effort to detect the presence of any viral agent. PPLO were isolated from every group with sinusitis. All virus isolation attempts were negative.

Ten turkeys were inoculated in the infraorbital sinus with 0.5 ml of undiluted allantoic fluid containing the "Crawley virus" and observed for 30 days. No symptoms or lesions of sinusitis developed.

In three separate attempts the "Crawley virus" survived through three, but not four, cultural transfers in PPLO media.

From this study it appears that PPLO are the primary etiological agent of infectious sinusitis of turkeys. One culture of PPLO reproduced the typical disease after 43 cultural transfers. No known avian viral agent will survive in cell free media at a temperature of 37.5 C for this period of time. The virus isolation studies on the turkeys which developed sinusitis served to elminate any of the agents that can be recognized by propagation in chicken embryos.

Apparently strains of PPLO vary widely in their ability to produce sinusitis in turkeys. Under the conditions of this study, 6 of the 12 strains isolated were considered non-pathogenic.

This study indicates that PPLO tend to lose their pathogenicity when serially transferred in carbohydrate media. This fact, along with the existence of relatively non-pathogenic strains, probably explains the difficulty some workers have experienced in producing sinusitis in turkeys with cultures of PPLO.

Although the "Crawley virus" has been suggested as a possible primary or contributing cause of infectious sinusitis, this study indicates that it plays no part in the cause of this disease. A known strain of this virus caused no recognizable disease condition when inoculated into the sinus of turkeys. Because of the characteristics of this virus in chicken embryos, it should be easily detected. Yet, on numerous attempts, it was not isolated from turkeys with gross lesions of infectious sinusitis.

SUMMARY

Twelve strains of PPLO were isolated and their ability to cause infectious sinusitis in turkeys was determined. Five strains were from turkeys and 7 were from chickens. Three of the turkey strains and two of the chicken strains caused a grossly visible sinusitis in turkeys. Two strains from turkeys and 5 from chickens failed to produce a grossly recognizable infection in turkeys under the conditions of this study. No viral agent was demonstrated from any case of sinusitis. PPLO were isolated from each group that developed the disease.

A known strain of "Crawley virus" failed to cause a recognizable infection in ten turkeys inoculated in the sinus with undiluted allantoic fluid containing the virus. Furthermore this virus was not isolated from any group of turkeys with gross lesions of infectious sinusitis.

In three separate attempts, the Crawley virus survived three, but not four, cultural transfers in carbohydrate media enriched with PPLO serum fraction and incubated at 37.5 C.

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AVIAN VIBRIONIC HEPATITIS

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IN DECEMBER 1955, a vibrio was isolated from the liver of an adult White Leghorn. The history of the flock revealed that production had been 15 to 20 percent below the expected level for several months. Also there was an increasing number of cull birds manifesting pale, dry and shrunken combs. On necropsy, the surface of the liver was found to be studded with hemorrhages varying from two to three millimeters in size. The ovary had regressed and only a small cluster of pea size ova remained. The kidneys were pale and slightly enlarged. A small block of liver tissue approximately 0.5 cm. square was aseptically collected, suspended in about five times its volume of nutrient broth and triturated. Four, six-day embryos were inoculated into the yolk sac with 0.2 ml liver suspension. All the embryos died the second day. Yolk smears from these embryos, stained with Gram stain revealed Gram negative vibrios. Yolk from subsequent embryo passages was lyophilized. Propagation of the organism was accomplished by inoculating a yolk suspension of the vibrio on to chicken infusion agar and incubating under 10 percent carbon dioxide tension. Growth was obtained more readily with yolk than allantoic fluid. At this time, no relationship was suspected between the hepatitis agent isolated by Delaplane

et al.[1] and the vibrio we recovered. Delaplane *et al.*[1] in describing their agent had reported that, "the infective agent was subjected to aerobic and anaerobic methods of culture, without success."

Cultivation of the vibrio: Since this original isolation of vibrio, a total of 29 isolations have been made from 14 different flocks. Isolations of vibrio have been made from chickens 8 weeks of age and older. At first, successful isolations were made from livers, and in one instance ovarian tissue, that were suspended in nutrient broth in approximately a 1:5 dilution, triturated in a Ten Broeck grinder and inoculated in 0.2 ml amounts into the yolk sac of 5 to 7-day embryos. When isolations were successful, embryo mortality started 3 to 5 days post inoculation. Dead embryos had a marked congestion of the dermal tissues. Embryos dying in the last trimester of incubation had enlarged livers that were mottled or studded with focal areas of necrosis. The spleens were enlarged. The myocardium was pale. In embryo assays, the vibrio was inhibited by streptomycin at the level of one mg/ml of tissue suspension and death of the embryos was delayed by penicillin in a concentration of 10,000 units/ml of tissue suspension. The embryo lethal dose (ELD) of the yolk from embryos dying following inoculation was 10^{-5} to 10^{-6} in five day embryos.

As studies with this organism progressed, attempts were made to isolate the vibrio on artificial media directly from the tissues of field specimens. Isolations of vibrios from clinical cases of hepatitis in chickens were made by aspirating bile from the gall bladder and inoculating a few drops on blood agar plates. The plates were incubated at 37.5 C in a Brewer anaerobic jar containing approximately 10 percent CO_2, 63 percent methane and 27 percent air. Isolations were also made under reduced oxygen tension by using 10 percent CO_2 in the Brewer jar. After 18 to 24 hours incubation, despite the fact that no colonies were visible, a smear of the agar stained with basic fuchsin or safranin for three minutes revealed large numbers of vibrios.

TABLE I

Summary of successful vibrio isolations by embryo and blood agar inoculation from approximately one hundred attempts

				Tissues cultured* no. positive /no. cultured	
Acc. no.	Breed	Age	% production	Liver†	Bile§
55511	W.L.	12 mos.	60	1/1	
56666	B.R.	9 mos.	40	1/1	
56693	B.R.	9 mos.	45	4/4	
56694	R.I.R.	Adult	40	2/2	
56715	W.L.	7 mos.	60	1/1	
56736	W.L.	8 mos.	70	3/3	
56769	W.L.	9 mos.	46	2/2	
56769	W.L.	7 mos.	35	2/2	
56832	W.L.	1 year	50	2/3	
56862	W.L.	1 year	50	2/3	
56985	W.L.	1 year	60	1/1	
57167	W.L.	14 mos.	50	2/3	
57621	W.L.	12 mos.	70	1/1	
57649	W.L.	12 mos.	70	3/6	
57691	W.L.	7 mos.	?	1/1	
57765	W.L.	10 mos.	45	1/1	
57771	W.L.	7 mos.	30	1/1	1/1
57796	W.L.	12 mos.	?		1/1
57812	W.L.	8 mos.	55	1/1	0/1
57828	W.L.	7 mos.	70		2/5
57854	W.L.	10 mos.	70	0/1	3/5
57854	W.L.	12 mos.	60		
57951	W.L.	17 mos.	?		1/1
58004	W.L.	10 mos.	80		1/1
58006	W.L.	Adult	30		1/3
58030	W.L.	12 mos.	50		1/1
58081	W.L.	17 mos.	50		1/1
58093	W.L.	12 mos.	?		3/3
58306	W.L.	8 wks.			1/1

*Three isolation attempts from spleens were negative. An isolation of vibrio was made from the ovary of one bird in accession 56832.
†Isolated by yolk sac inoculation of 5 to 7 day embryos.
§Isolated on blood agar under reduced oxygen tension.

If incubation was continued for three days, a mucoid growth was visible (figure 1). Direct smears of bile were stained but only coccoid forms could be seen. One could not be certain that these coccoid forms were vibrios or extraneous material.

Some strains of vibrios were readily subcultured on artificial media whereas other strains were exceedingly difficult to subculture and in some cases the organisms died out after three or four transfers. As the organism became adapted to blood agar under reduced oxygen, colonies formed

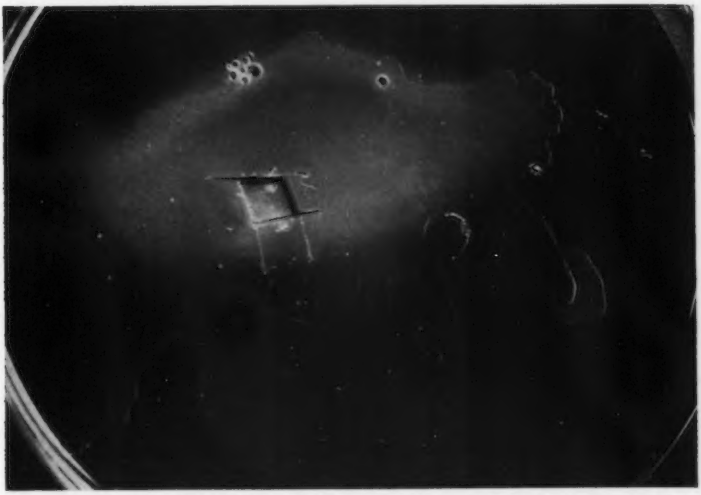

Fig. 1. Seventy-two hour culture of vibrio growing under reduced oxygen tension on blood agar inoculated with bile. Note mucoid appearance.

after 24 and 48 hours of incubation (figures 2 and 3). Successful propagation of these vibrios has been accomplished under reduced oxygen tension on chicken infusion agar, blood agar, Bacto-thiol, thioglycollate with serum and PPLO liquid media with serum fraction without inhibitors. Growth did not occur in the presence of thallium acetate.

Characteristics of the vibrio: Stained smears from agar reveal this vibrio as short, comma forms, "S" shaped forms or long spiral forms 5 to 12 microns in length (figures 4, 5, 6). In yolk smears, the organism appears as comma or "S" shaped forms. Many times it is extremely difficult to see any characteristic vibrio forms in yolk smears. Smears of allantoic fluid show S shaped and spiral forms (figure 6). Older cultures on agar tend to produce coccoid forms.

Lyophilized vibrios remained viable for twenty months (longest period tested). Yolk suspensions in a deep freeze have remained viable for ten months. The organism in tissues and bile remains viable for at least six days at refrigerator temperature.

The vibrio was inhibited by oxytetracycline 10 micrograms, chlortetracycline 10 micrograms, streptomycin 10 micrograms, and furadantin 50 micrograms, when applied in sensitivity discs. Slight inhibition was demonstrated by polymyxin B sulfate 10 micrograms. Penicillin (1.5 units) had very slight inhibitory effect and bacitracin (5 units) none (figure 7).

In filtration studies, the vibrio was retained by Berkfeld V and N candles and by Seitz EK pads. The organism in a 10 percent yolk suspension in nutrient broth passed a millipore filter disc of 0.45 micron porosity. Vibrio washed from the surface of an agar plate with nutrient broth also passed through the .45 micron millipore filter.

Miss E. O. King[3] in studying our organism, found it to have many characteristics of a vibrio which had been isolated from humans. The following is a summary of characteristics as determined by Miss King: glucose-, xylose-, mannitol-lactose-, sucrose-, maltose-, MacConkey+light, S.S.-, hemolysis-, citrate-, urea-, nitrate-, indol-, H_2S TSI paper 2+, butt-, MR/VP-/-, gelatin-, litmus milk-, short, curved rods and spiral forms, gram-, spores-, O_2 req. microaerophilic, temp. 37 to 42 C, pigment slight yellow, oxidase+, motility+, catalase 2+.

Pathogenicity of the vibrio: In an effort to determine the pathogenicity of this vibrio for chicks, inoculation trials were made. Initial trials were done with yolk suspensions inoculated intramuscularly in 0.2 ml amounts into one-week-old chicks. No chicks died following inoculation. At necropsy 7 to 10 days post inoculation, the livers were enlarged and studded with focal necrosis. The spleens were enlarged. There was a marked paleness of the myocardium and the pericardial sac was distended with a clear amber fluid. Reisolation of the vibrio was accomplished from the livers and pericardial fluid of these chicks. Inoculation of turkey poults produced similar lesions.

In an effort to establish the fact that a pure vibrio culture was infectious for chicks, additional trials were done. A yolk suspension of vibrio isolated from a field case was serial-

Fig. 2. Forty-eight hour culture of vibrio on blood agar showing discrete colonies.

Fig. 3. Forty-eight hour culture of vibrio on blood agar plate that had been heavily seeded from another culture. Note discrete colonies emerging from the gray film of confluent growth.

ly cultured for four passages on blood agar incubated at 37.5 C under 10 percent CO_2 and under 63 percent methane. This was done to free the yolk suspension of vibrio from any accompanying viral agent. The fourth passage vibrios were then washed off the agar plate and 10^{-5} ELD were inoculated intramuscularly into 18, one-week-old chicks. Previous isolation attempts in embryos from livers of chicks from the same hatch had failed to yield the organism. Seven to 10 days later, the inoculated chicks were necropsied. Gross lesions similar in appearance to those in the yolk inoculation trials were present but in fewer numbers and smaller in size. Vibrios were reisolated from these chicks. The histological appearance of hearts, livers and spleens was identical to that described by other workers for avian infectious hepatitis.[1,7,8,9,10] Cultures that had undergone only one passage on artificial media or in embryos were more virulent for chicks than those that had been repeatedly transferred.

Different strains of hepatitis agent in yolk received from two other laboratories contained vibrios. These organisms were demonstrated by direct smear of the yolk and also by in vitro culture.

In one trial, inoculation of Swiss white mice intracerebrally produced nervous symptoms and the organism was reisolated from the brain. However, the agent was not uniformly pathogenic for mice.

DISCUSSION

Hofstad[2] also reported the isolation of a vibrio from diseased livers of adult chickens. It is apparent that the condition heretofore described by other workers[1,2,7,8,9,10,11] and avian vibrionic hepatitis are caused by a similar, if not identical, agent. The clinical and pathological manifestations of vibrionic hepatitis are identical with those described for a hepatitis caused by an unknown agent.[1,7,8,9,10] The sensitivity to antibiotics of the avian vibrio is comparable to that described for the hepatitis agent.[1,7,8,9,10,11] The results of filtration studies with avian vibrio are similar to those described by Winterfield.[11] Embryo inoculation studies with cultures of vibrio from artificial media gave a death pattern and embryo lesions

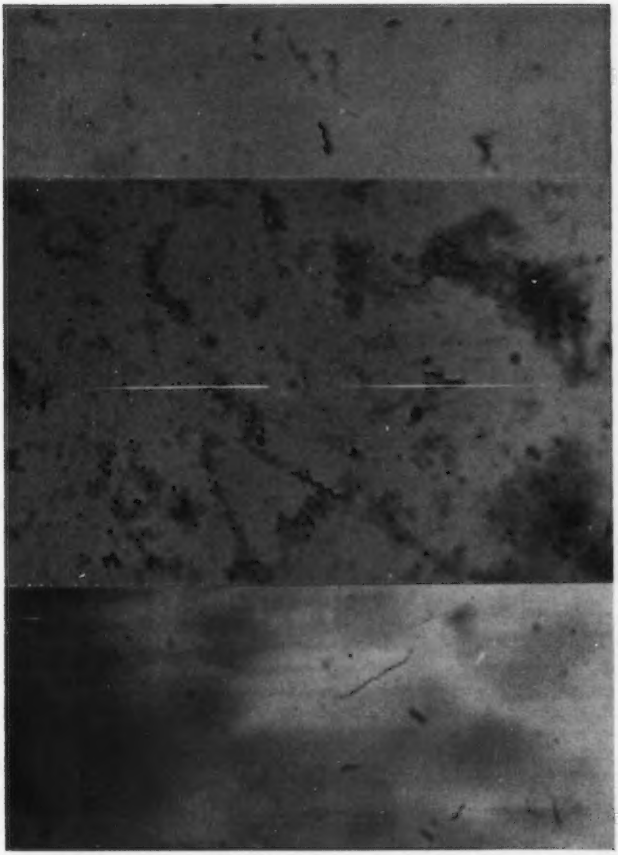

Fig. 4. (top) Smear from 24 hour culture grown on blood agar after inoculation with yolk suspension. Note comma and S forms. Basic fuchsin ×900.

Fig. 5. (middle) Smear from 72 hour culture grown on blood agar after inoculation wtih yolk. Note spiral forms, comma forms and coccoid forms. Basic fuchsin ×900.

Fig. 6. (bottom) Smear of allantoic fluid with long spiral form. Basic fuchsin ×900.

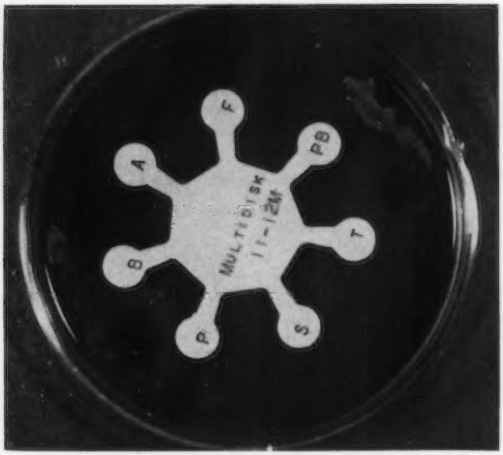

Fig. 7. Blood agar plate after 48 hours incubation showing sensitivity of vibrio to A-aureomycin, F-furadantin,, PB-polymyxin B sulfate, T-terramycin, S-streptomycin, P-penicillin and B-bacitracin.

in the liver, spleen and heart identical with those previously reported.[1,2,7,8,10,11] Chick inoculation trials with vibrio cultured on artificial media produced gross and microscopic lesions identical with those as being conceived as due to an agent.[1,2,7,8,10,11]

Avian vibrionic hepatitis appears to be different from the virus hepatitis of birds described by Lucke.[4] Dr. Ratcliffe, in a personal communication, stated that gallinaceous birds were refractory to virus hepatitis. This is in marked contrast to vibrionic hepatitis.

Marthedal[6] reported a hepatitis in chickens accompanied by typhlitis. In avian vibrionic hepatitis, no typhlitis was ever found in natural or experimental cases. It would appear that the condition reported by Marthedal and avian vibrionic hepatitis are dissimilar.

The organism reported by Lukas[5] as causing a hepatitis and the vibrio described in this paper have dissimilar characteristics. The organism described by Lukas was non-motile,

grew in the presence of thallium acetate and its morphology varied from globular to a filamentous, branching body.

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SUMMARY

A vibrio has been repeatedly isolated from the livers and bile of adult birds submitted from flocks with histories and gross lesions of hepatitis. Inoculation of this vibrio into young chicks produced hydropericardium, paleness of the myocardium, enlarged spleens and focal necrosis in the liver. The vibrio was reisolated from the inoculated chicks. Isolation of the vibrio has been accomplished by inoculation of the yolk sac of 5 to 7-day embryos with liver, spleen, pericardial fluid and bile from inoculated chicks. Vibrios have been isolated from clinical cases by the inoculation of bile on blood agar which was then incubated under reduced oxygen tension. Vibrios similar to those isolated in this laboratory have been demonstrated in yolk material containing the hepatitis agent sent to us from two other laboratories. In view of the evidence presented, it is proposed that the clinical, pathological syndrome caused by a vibrio be designated avian vibrionic hepatitis to distinguish it from other hepatitides.

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AVIAN INFECTIOUS HEPATITIS

By M. S. HOFSTAD, E. H. McGEHEE, and P. C. BENNETT

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IN JANUARY 1956, cases of severe hepatitis were brought to the Iowa Veterinary Diagnostic Laboratory from a flock of laying chickens which had experienced some mortality and lowered egg production. Routine cultures of the liver re-

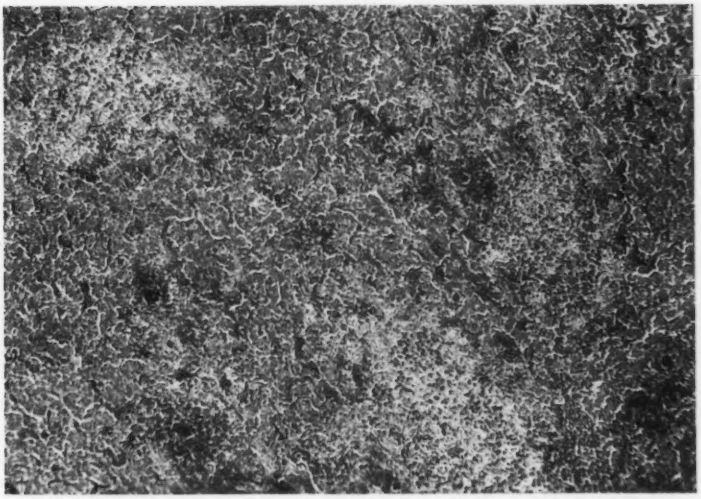

Fig. 1. Chicken liver, natural case of infectious avian hepatitis (2063A). Congestion, areas of necrosis containing numerous neutrophiles and some areas of lymphocytic infiltration are apparent. Hematoxylin and eosin. ×96.

vealed no pathogenic organisms. However, suspensions of liver tissue inoculated into the yolk sac of 7-day-old chicken embryos caused death of the embryos in 4 to 6 days. Serial passages were accomplished with yolk collected from dead embryos. Additional isolations of the agent were made from cull birds of another flock in which the liver lesions were less severe.

Liver pathology: The gross pathology was variable. In the initial cases, the livers were enlarged, mottled, and had irregular, white areas of necrosis. In the experimentally inoculated birds and in other naturally occurring cases, the livers were of normal size and color but had small, necrotic areas frequently in only a portion of one lobe.

Microscopically the liver section had general congestion, localized hemorrhages, large areas of necrosis with the liver structure completely gone and infiltrated with erythrocytes, neutrophiles and lymphocytes (figure 1).

Studies with the agent: Yolk or yolk sac membrane from embryos that had died had a titer of $10x10^{5.0}$ e.l.d.$_{50}$ or higher per ml when inoculated into the yolk sac of 6 or 7-day-old chicken embryos. Most of the embryos died on the fourth or fifth day and lesions consisted primarily of congestion of the yolk sac and embryo. Liver lesions were not discernible at this age. However, when older embryos were inoculated via the allantoic sac with infective yolk from dead embryos, enlarged spleen and marked liver necrosis were the most outstanding lesions (figure 2). Several experiments may be cited to illustrate this effect on older embryos.

Fifteen-day-old embryos were given 0.1 ml dose into the allantoic sac of a 10^{1} and 10^{2} dilution of infective yolk. Many of the embryos were dead on the 20th day and the primary lesions were enlarged and necrotic livers and enlarged spleens. In a similar experiment with 15-day-old embryos and a different inoculum, 18 out of 52 embryos failed to hatch, and most of these had extensive lesions. Even some of those that hatched and appeared normal had liver necrosis. Other experiments using 11 and 13-day-old embryos resulted in variable mortality, and the embryos which died usually had extensive liver damage and an enlarged spleen. In one of these involving 13-day-old embryos inoculated via the allantoic sac, all but a few of the 29 embryos were dead at hatching time and the majority of these had extensive liver damage. In another experiment, a liver suspension from an infected embryo was titrated in 11-day-old embryos via the allantoic sac route with 0.2 ml dose. The highest dilution inoculated was the 10^{-4}. On the 21st day, two embryos were dead and two were alive that had been inoculated with this dilution. All four embryos had liver necrosis and enlarged spleens.

Filtration and antibiotic sensitivity of the agent: A 1:25 dilution of yolk from the third embryo passage of the infective agent was found to pass through a Selas 02 porcelain filter, consistently, but not the Selas 03. In one of three trials the agent did pass the 03 Selas. This might indicate that the agent is relatively small and comparable to the size of *Mycoplasma gallinarum*.

Fig. 2. Two chicken embryos, 19 days, (right and left) infected with yolk passage infectious hepatitis agent. Note liver enlargement and necrosis. Middle embryo is normal.

Antibiotic sensitivity tests were conducted in 7-day-old embryonated chicken eggs inoculated with approximately a 1:15 dilution of infected yolk material. The mixtures of yolk and antibiotic were held at room temperature 30 minutes before inoculation.

The agent was susceptible to 0.5 mg/ml of streptomycin. It was resistant to penicillin except in large doses. In one instance, isolation of the agent was accomplished from an untreated liver suspension, but was not from a penicillin treated liver suspension. When 2000 u/ml of penicillin was used the agent was not destroyed but embryo mortality was delayed a few days. The agent withstood 5000 u/ml of bacitracin, but was susceptible to 2.5 mg/ml of terramycin and 5 mg/ml of aureomycin. One strain was slightly more resistant to the last two antibiotics. The agent was also susceptible to thallous acetate.

Viability of the agent on storage: The agent withstood storage in the freezer at —25 C for at least 2 years. Infective yolk material at the end of 1 year had at least a titer of $10x10^4$e.l.d.$_{50}$/ml. Infective material lyophilized and stored at 4 C remained viable for 6 months, the longest period tested.

Growth of the agent in cell free medium: Attempts to grow the agent in chicken infusion medium[2] containing 20 percent chicken serum were successful when the inoculum was allantoic fluid and diluted yolk from dead, infected embryos.[3] The agent grew in granular masses which usually settled out on the sides and bottom of the tube. Some difficulty was encountered in preparing consistently satisfactory medium, but when a good batch of medium was prepared, isolation of the agent was readily accomplished.

Stained smears of sedimented cultures usually reveal masses of coccoid organisms and many vibrio (figure 3). The organism is motile, stains readily with methylene blue and Giemsa's stain and is Gram negative. Occasionally smears may reveal some spiral-shaped organisms. The coccoid masses apparently represent older elements and are found exclusively in older cultures. The vibrios represent young, reproducing organisms and predominate in 24 to 48 hour cultures. Eleven-day-embryos inoculated into the allantoic sac died and yielded allantoic fluid which was sedimented by slow speed centrifugation. Smears from the sediment revealed coccoid bodies.

The vibrio culture produced an identical mortality pattern in 7-day-old embryos even after many transfers. For example, after the 31st culture transfer, a 1:10 dilution of culture killed all embryos on the 4th day. Yolk material saved from one of these embryos was titrated in 7-day-old embryos and gave a typical titer and mortality pattern. However the culture had apparently lost some virulence for older embryos since very few died with typical hepatitis following allantoic sac inoculation.

DISCUSSION

The hepatitis in chickens observed in Iowa appears to

Fig. 3. Seventy-fifth culture transfer of isolated organism. Giemsa stain. ×1590.

be similar to that described by Delaplane *et al.*,[1] Moore,[5] Winterfield *et al.*,[9,10] Sevoian *et al.*[7] and Peckham.[6] Moore[5] indicated that studies of tissue sections furnished by Tudor[8] revealed that the hepatitis described by Tudor was probably the same as that described by Delaplane *et al.*[1] It does not appear that the hepatitis described by Lukas[4] is related to the hepatitis described here.

Strains of the hepatitis agent described by Winterfield *et al.*[10] were obtained through the courtesy of Dr. R. W. Winterfield. These strains appeared similar to the Iowa strains with respect to embryo pathogenicity, lesions and mortality. The antibiotic sensitivity tests were essentially similar except one of his strains (9805) was slightly more resistant to aureomycin and terramycin. These strains also yielded vibrio organisms on culture in artificial medium.

SUMMARY

In January 1956, cases of hepatitis were diagnosed in

two flocks of chickens. In one flock there was some mortality while in the other only a few culls were observed to have hepatitis. An agent, lethal for 7-day-old chicken embryos, was isolated. The disease could be reproduced in chickens by parenteral inoculation and the agent reisolated from the livers of the inoculated chickens. A vibrio organism was isolated in artificial medium from infected embryos which, after many passages, produced a typical mortality pattern in inoculated chicken embryos.

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ANTIBODY RESPONSE OF THE CANADA GOOSE TO THE NEWCASTLE DISEASE VIRUS*

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NEWCASTLE disease has a world wide distribution[2] and it has been found to infect a wide variety of birds including chickens,[5] pheasants, quail, partridge, pigeons,[1] and doves[7] to name just a few. No evidence of infection of the Canada goose (*Branta canadensis*) by this virus has as yet been reported. However, the susceptibility of this bird species to the virus has become more important in view of the changes in its wintering habits.[8] Canada geese were at one time wintering over a widespread area along the coastal region of the Gulf of Mexico. In recent years, however, 100,000 to 200,000 birds have concentrated at Horseshoe Lake, Illinois or in the near vicinity.[6] This crowding has increased the chances of exposure of this bird species to respiratory diseases of all types.

EXPERIMENTAL PROCEDURES

All birds used in this study were trapped by the refuge

*This study was supported by a research grant, E-1029, from the National Institutes of Health, Public Health Service.

staff, Horseshoe Lake Refuge, Illinois and shipped immediately by truck to this laboratory. Each bird was tagged for identication and a 2 ml blood sample was taken by cardiac puncture. Serum harvested from each blood sample, was checked for bacterial sterility and stored at 4 C. These sera represented pre-exposure samples for this and subsequent experiments. The geese were maintained in three holding pens separated from any other domestic poultry by a minimum of 150 feet. All birds were fed a ration of corn and all purpose chicken feed. Any unconsumed feed was discarded each day to minimize fungus contamination.

The Newcastle disease virus (NDV) used in this study was originally isolated from lung and tracheal material from naturally infected chickens, and cultivated in the allantoic sac of embryonated chicken eggs. It consisted of pooled allantoic fluid taken from embryos dead by the second day following inoculation of 10-day embryos via the allantoic cavity route. The pooled virus suspension was stored at a —20 C. Following thawing, it was processed by centrifugation at 2500 rpm in a PR-1 International centrifuge for 15 minutes to remove the insoluble precipitate formed upon thawing. This virus was capable of killing all chicken embryos within 48 hours after inoculation.

Ten geese were exposed to 0.3 ml of NDV suspension by intra-nasal inoculation, and 0.3 ml of NDV suspension by intra-tracheal inoculation. Ten ml of blood were taken by cardiac puncture from each bird just preceding the virus inoculation and also at 1, 2, 3, 4, 6, 8 and 10 weeks post- inoculation. After clotting and serum elution, the specimen was centrifuged at 1500 rpm to express additional serum. The serum was harvested, tested for bacterial sterility and stored at 4 C until used. All sera in this experiment were diluted ten fold in sterile nutrient broth. The results of the serum neutralization tests were adjusted accordingly.[9]

All geese surviving the experiment at the 10th week were challenged with a 0.3 ml intra- nasal inoculation and a 0.3 ml intra-air-sac inoculation of NDV. Serum samples were taken at 1, 2 and 3 week intervals following the second virus

exposure. These sera were pooled, checked for bacterial sterility and stored at 4 C.

Serum neutralization tests were run on all serum samples according to the procedure outlined by Cunningham.[4] Five 10-day embryos were used for each dilution. All inoculated embryos were examined by trans-illumination 16 hours after inoculation and then daily for four additional days. Deaths during the first 16 hours following inoculation were considered to be non-specific and were not considered in the final results. All results were evaluated according to the ld_{50} procedure of Reed and Muench.[10]

RESULTS

Pre-exposure serum samples from 33 geese were checked for antibodies (table I). The average neutralization index (NI) for these sera was found to be $10^{1.18}$. Thirteen samples had a NI of $10^{0.0}$ or less; nine samples had a NI of $10^{1.0}$ to $10^{1.8}$; and, eleven samples had a NI of between $10^{2.0}$ and $10^{2.5}$. The antibody titer expressed as the NI for each post-inoculation period is presented in table II. Generally, the highest antibody response occurred between the first and second post-inoculation period with the high titers for all birds ranging between $10^{3.8}$ and $10^{5.5}$ The NDV neutralization capacity of the anti-serum rapidly declined and reached an average titer of $10^{0.2}$ by the tenth week.

A sharp rise in neutralization capacity resulted from a second exposure to NDV with a high titer of $10^{5.9}$ on the pooled serum after one week. By the third week the titer had decreased to $10^{4.0}$. Careful examination of all birds throughout the course of the experiment did not reveal any of the typical symptoms associated with Newcastle disease. No other evidence of illness was detected. The experiment was terminated at this point due to the lack of experimental animals.

DISCUSSION

Approximately 60 percent of the birds studied in this experiment had titers higher than has been reported for normal

TABLE I

Pre-exposure NDV antibody titers of the Canada goose

No. of birds	Neutralization index*
1	0.0
5	0.1
2	0.2
1	0.3
2	0.5
2	0.8
1	1.0
2	1.2
3	1.3
2	1.4
1	1.8
5	2.0
3	2.1
1	2.2
1	2.4
1	2.5

* Expressed as the exponent of the $\log_{10}$.

TABLE II

Antibody response following exposure to Newcastle disease virus at 0 weeks and 10 weeks

Goose no.	Neutralization index* Time in weeks 0	1	2	3	4	6	8	10	11	12	13
1	1.0	3.9	2.5	2.9	2.5	2.8	0.3	0			
2	0.2	2.2	2.0	2.3	1.4	0	0.3	0		..	
3	0.8	3.1	3.0	3.7	1.4	1.3	0	0			
4	1.4	2.8	4.4	2.1	2.3	2.8	0.3	0			
5	0.8	3.8	2.7	1.3	1.0	0	0	0.3			
6	2.0	2.7	3.2	1.5	2.1	0	0.3	0.1			..
7	1.2	3.8	3.2	3.0	1.5	0	0.3	0			
8	1.3	5.5	5.2	3.0	3.2	2.1	3.3	1.3			
9	2.5	4.5	4.2	3.0	3.0	1.4	0	0			
10	2.2	4.3	5.4	3.3	2.0	0	—	—	5.9†	5.3†	4.0†
Average	1.3	3.7	3.6	2.6	2.0	1.0	0.5	0.2	5.9	5.3	4.0

*Expressed as the exponent to $\log_{10}$.
†Represents titer of pooled sera.

non-exposed chickens. However, since titers of serum from known unexposed Canada geese were not available, it would be unsafe to interpret our findings on the basis of available data for chickens. The relatively poor response of goose No. 2

to the first exposure of Newcastle disease might cause one to conjecture that this bird might not have experienced an exposure prior to trapping and that the response of Canada geese is relatively weak to Newcastle virus. The same might be said for goose No. 6 despite the fact that its initial titer was 10^2. This poor initial response might cause one to believe that 10^2 might be a normal, non-immune serum titer. Certainly, if the intial titer of goose No. 6 were the result of a previous exposure, one would expect a higher gain in titer afer the first experimental exposure than the observed increase of only 1.2 logs.

SUMMARY

The sera of 33 wild Canada geese were examined for SN titers to Newcastle disease. The average SN titers was 1.2 with the titers ranging from 0 to 2.5. Nineteen birds had titers greater than 1.0. When ten of the birds were exposed to Newcastle disease virus of chicken orgin, no symptoms or deaths occurred. Serum titers increased from 1.2 to 4.2 logs within two weeks. By ten weeks titers virtually dropped to zero.

A second virus exposure at ten weeks produced a prompt increase in average titers to 5.9 after one week.

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CASE REPORT—ISOLATION OF THE AGENT OF INFECTIOUS SINUSITIS OF TURKEYS FROM NATURALLY INFECTED PHEASANTS

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Received 21 Feb 1958

THE purpose of this paper is to describe a naturally occuring transmission of the infectious sinusitis agent (*Mycoplasma gallinarum*) from turkeys to pheasants. Jungherr *et al.*[1] in 1952 reported that the pheasant was susceptible to the CRD agent. Van Roekel *et al.*,[2] in 1953, was able to infect pheasants both by inoculation and by contact exposure to artificially infected pheasants. Van Roekel *et al.* also observed that intrasinus, intratracheal, and air sac inoculation but not intramuscular inoculation, produced a disease syndrome in the pheasant similar to that observed in artificially infected chickens.

A review of the literature indicates that this is the first report of a naturally occurring outbreak of the infectious sinusitis agent in pheasants. In August, 1957 an outbreak of infectious sinusitis occurred in a market flock of white turkeys on a ranch just north of St. Paul. The flock consisted of 1650 White Holland turkeys, most of which had respiratory rales and approximately 20 percent had enlarged infraorbital sinuses.

Across the road from this flock was a pen of 300 pheasants which were brooded in the same building with 200 poults from the same hatch as the infected flock. The same

* Technical assistance by Mrs. Colvard.

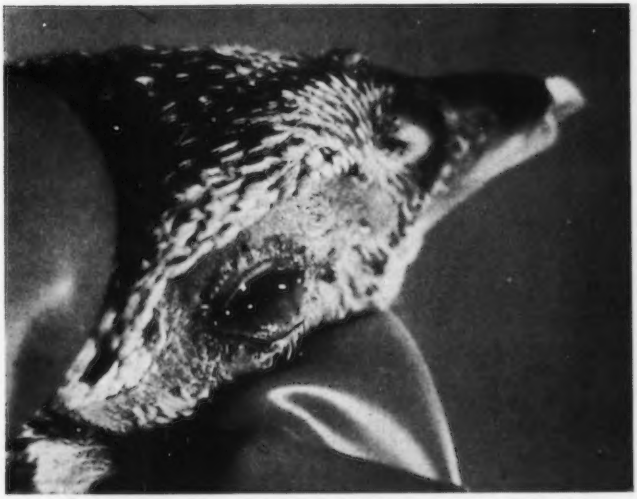

Fig. 1. Pheasant infected with PPLO. Note marked thickening of the eyelid accompanied by extensive lacrimation.

caretaker fed and watered both the infected turkey flock and the pheasants. The outbreak of the disease first occurred in the turkeys when they were 5 weeks of age and then spread to the 7-week-old pheasants. The mortality observed in the turkey flock was about 10 percent whereas the mortality of the pheasants was about 30 percent.

Both turkeys and pheasants were submitted to the laboratory for attempted isolation of the agent. The lesions found in the turkeys were the typical, enlarged, infraorbital sinuses and air sac lesions commonly associated with the infectious sinusitis agent. The pheasants also had enlarged infraorbital sinuses. However, little exudate was present within the sinuses. The eyelids of the pheasants were thickened and extensive lacrimation was noted. The air sacs were involved very slightly as only minute areas of infiltration were observed.

The turkey and the pheasant sera reacted to the PPLO serum plate agglutination test. A portion of the trachea at the bronchial bifurcation, air sac lesions and sinus exudate

were cultured in PPLO media. Cultures were made in duplicate. One set contained PPLO broth Wo/CV (Difco 0554-01) with 1 percent enzymatic yeast hydrolysate and 1 percent PPLO serum fraction, (Difco 0441-63). The other media consisted of phenol-red maltose broth (Difco 0093-01) with yeast hydrolysate and PPLO serum fraction at the same amount.

Mycoplasma gallinarum was isolated from both the turkeys and the pheasants. The isolate from turkeys fermented maltose without gas after three weekly subcultures. The isolate from pheasants did not ferment maltose phenol-red broth until after five weekly transfers. The isolate from pheasants was then inoculated into turkeys via the infraorbital sinuses, and produced enlarged sinuses in about 8 days. Serum collected from the inoculated turkeys became positive to the PPLO serum plate test. The organism was then re-isolated from the enlarged turkey sinuses. The organism after reisolation from the turkeys fermented PPLO maltose after three weekly subcultures. The isolate from pheasants was also inoculated into other pheasants and was observed to produce a disease syndrome similar to that observed in the natural outbreak.

The isolate from turkeys was inoculated into pheasants and produced symptoms and lesions in the pheasants similar to those observed in the natural outbreak. When the agent was reisolated from the pheasants it again required five weekly subcultures to ferment PPLO maltose.

Both the turkey and the pheasant isolates were found to be lethal for 7-day-embryonated chicken eggs when inoculated by the yolk sac route.

SUMMARY

A transmission of *Mycoplasma gallinarum* from turkeys to pheasants occurring under natural conditions is reported. The agent, when isolated from pheasants and inoculated into turkeys was found to produce infectious sinusitis.

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1 UNIVERSITY OF GEORGIA FEEDING TRIAL

Treatment (40 birds per treatment)	Antibiotic Per Ton of Feed (grams)	Av. Wt. 8 Weeks (lbs.)	Feed Conversion
BACIFERM (zinc bacitracin)	4	2.61	2.13
Penicillin	4	2.58	2.12
BACIFERM-PB*	4	2.69	2.23

*contains zinc bacitracin plus penicillin (3:1 ratio)

2 UNIVERSITY OF MASSACHUSETTS TEST

Treatment (32 birds per treatment)	Antibiotic Per Ton of Feed (grams)	Average Male Wt. 8 Wks. (lbs.)	Lbs. Feed per Lb. Gain	Mortality (each treatment 32 birds started)
Control	0	3.26	2.15	1
BACIFERM (zinc bacitracin)	4	3.34	2.16	0
BACIFERM (zinc bacitracin)	6	3.38	2.11	1
Penicillin	4	3.37	2.11	2

3 ALABAMA POLYTECHNIC INSTITUTE TEST (No. 2)

Treatment (40 birds per treatment)	Antibiotic Per Ton of Feed (grams)	Av. Wt. 7 Wks. (lbs.)	Lbs. Feed per Lb. Gain
BACIFERM (zinc bacitracin)	4	2.60	2.00
Penicillin	4	2.53	2.04

4 LEADING NORTHEASTERN UNIVERSITY

Treatment (100 birds per treatment)	Antibiotic Per Ton of Feed (grams)	Av. Wt. 9 Weeks (lbs.)	Lbs. Feed per Lb. Broiler
Control	0	3.43	2.44
BACIFERM (zinc bacitracin)	4	3.49	2.40
Penicillin	4	3.40	2.40

5 FEED MANUFACTURER IN SOUTHEAST

Treatment (200 birds per treatment)	Antibiotic Per Ton of Feed (grams)	Av. Wt. 66 Days (lbs.)	Lbs. Feed per Lb. Gain	Cost per Lb. Meat
Penicillin	4	3.18	2.31	14.73¢
BACIFERM (zinc bacitracin)	4	3.19	2.30	14.66¢
BACIFERM-PB*	5	3.19	2.30	14.70¢

*contains zinc bacitracin plus penicillin (3:1 ratio)

6 COMMERCIAL TEST FARM BROILER TRIAL

Treatment (1000 birds per treatment)	Antibiotic Per Ton of Feed (grams)	Av. Wt. 9 Weeks (lbs.)	Lbs. Feed per Lb. Gain
BACIFERM (zinc bacitracin)	4	3.2	2.48
Penicillin	4	3.2	2.48

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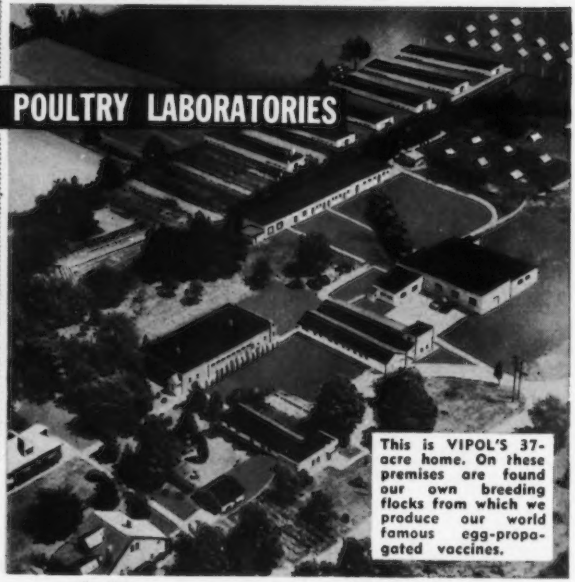

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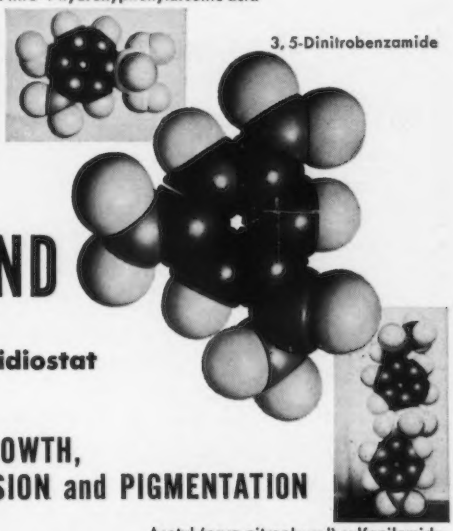

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Cuckler, A. C., and Malanga, C. M.: Proc. Soc. Exp. Biol. and Med. 92: 485, 1956.

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